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PTHrP was originally discovered as the tumor product responsible for the paraneoplastic syndrome of humoral hypercalcemia of malignancy. Recent experiments have shown that PTHrP has an important role as a developmental regulatory molecule. This project is designed to explore the hypothesis that PTHrP is a locally produced growth inhibitor that helps to regulate mammary development. In the first year we concentrated on exploring the consequences of the disruption of the PTHrP gene on mammary development. We found that PTHrP was necessary for embryonic mammary development. In the absence of PTHrP, the embryonic mammary gland failed to initiate branching morphogenesisand the mammary epithelial cells degenerated. We found that these effects were due to the actions of amino-terminal PTHrP acting via the PTH/PTHrP receptor. Furthermore, it appears that PTHrP is produced by the mammary epithelial cells and the PTH/PTHrP receptor is expressed on mammary stromal cells. Therefore our findings suggest that PTHrP serves as a critical signal from the embryonic epithelium to the embryonic mesenchyme in order to allow the mesenchyme to support further development and morphogenesis of the epithelium.

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FOREWORD

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INTRODUCTION

Parathyroid hormone-related protein (PTHrP) was originally discovered as the tumor product that is responsible for causing the clinical syndrome of humoral hypercalcemia of malignancy (HHM) (1). In this syndrome, PTHrP is released into the circulation by malignant cells, resulting in a typical constellation of biochemical abnormalities resembling hyperparathyroidism. The similarity of HHM and hyperparathyroidism is now understood on a molecular basis. The parathyroid hormone (PTH) and PTHrP genes arose by duplication from a common ancestral gene and continue to share a high degree of homology in their amino-terminal ends, a feature that allows them to signal through the use of a common receptor (termed the PTH/PTHrP type I receptor) (1,2). In the setting of malignancy, PTHrP, which normally acts as a local autocrine or paracrine factor, is secreted into the circulation by tumor cells and interacts with PTH/PTHrP receptors in bone and kidney, mimicking the actions of PTH (1).

As noted in the preceding paragraph, PTHrP normally acts as a local autocrine and/or paracrine factor. It is expressed in a great number of tissues, where it appears to play a role in the regulation of cellular proliferation and differentiation during development (3). One of these sights is the mammary gland. PTHrP mRNA has been shown to be expressed in the embryonic mammary epithelium, as well as by mammary epithelial cells during pregnancy and lactation (3,4). In addition, PTHrP has been shown to be secreted by mammary myoepithelial cells in culture (5,6). The PTH/PTHrP receptor is found on mammary stromal cells and on mammary myoepithelial cells (4-6). Therefore, the mammary gland contains the elements of both autocrine and paracrine signaling loops for PTHrP. That these loops are important to the physiology of mammary development is evident by the results of the overexpression of PTHrP in mammary myoepithelial cells (7). As reviewed in the original proposal, we used the human keratin-14 (K14) promoter to target PTHrP overexpression to myoepithelial cells. This resulted in a severe impairment of branching morphogenesis and mammary ductal proliferation during sexual maturation and pregnancy (7). These results led us to hypothesize that PTHrP acts as a local growth inhibitor, contributing to the regulation of ductal proliferation and morphogenesis during mammary development. The intent of the current grant is to test this hypothesis by examining the effects of PTHrP on branching morphogenesis and on mammary epithelial cell proliferation and transformation. In order to test this hypothesis we proposed a series of four technical objectives that encompassed a mixture of experiments *in vitro* and in transgenic animals. The following sections report our progress over the first year, organized by technical objective.

BODY

Technical Objective 1. - Effects of the Loss of PTHrP on Mammary Gland Development

Our first technical objective was to examine mammary development in the absence of PTHrP. We have concentrated most of our effort during the first year of this award on this specific aim. As described in the original proposal, we rescued the PTHrP-knockout mouse from its neonatal death (due to skeletal abnormalities, (8)) by breeding a bone-specific, PTHrP transgene (9) onto a homozygous, PTHrP-null background. This resulted in a mouse (termed the Col II-PTHrP/PTHrP-null mouse) that lacked PTHrP in all tissues except the skeleton where it was produced by virtue of a procollagen type II - PTHrP transgene. As described in the appended manuscript (10), these mice have a myriad of developmental abnormalities, but they survive to 3 - 6 months of age, which allowed us to examine their mammary gland development. Based on the results of PTHrP overexpression in the mammary gland (7), we had originally predicted that the lack of PTHrP would result in premature mammary development or mammary hyperplasia. Therefore, we were surprised to observe that these "rescued" PTHrP-null animals had no mammary glands. To be more precise, they had mammary fat pads that were devoid of mammary epithelium (see Fig. 1). Although not what we had originally predicted, we found this to be an exciting result, for it demonstrated that PTHrP was absolutely necessary for the proper development and survival of the mammary epithelial duct system, and we devoted much of the past year to characterizing this phenotype.

In addition to their lack of mammary epithelial ducts, Col II-PTHrP/PTHrP-null mice also lacked nipples. Because nipple formation in mice is tied to proper development of the embryonic mammary gland (11), the lack of nipples suggested to us that the failure of mammary development in these mice occurred before birth. In order to test this hypothesis we examined mammary development in the original PTHrP-knockout embryos. Embryonic mammary development involves two separate steps, the formation of the mammary bud and the initiation of branching morphogenesis which leads to the typical branched ductal pattern that is present at birth (see Fig. 2) (11). In the absence of PTHrP, the mammary buds appear to form appropriately, but at E16 the knockout mammary buds fail to elongate and initiate branching growth. Instead, the ducts become surrounded by an abnormally dense condensation of mammary mesenchyme and the mammary epithelial cells degenerate (Fig. 3). By birth, the PTHrP-knockout epithelium has disappeared.

PTHrP is a polyprotein and gives rise to several different biologically-active peptides each of which acts via its own receptor (3). We had previously found that the amino-terminal portion of PTHrP acting through the PTH/PTHrP receptor was responsible for the effects of PTHrP on branching morphogenesis during sexual maturation and pregnancy (7). In order to study if this same ligand/receptor pair was responsible for the effects of PTHrP on embryonic mammary development, we examined the effects of the loss of the PTH/PTHrP receptor on embryonic mammary development. Collaborating with Drs. Beate Lanske and Henry Kronenberg from Boston, we established a colony of

PTH/PTHrP-receptor knockout mice (12) and found that, identical to our previous results with PTHrP-knockout embryos, ablation of the PTH/PTHrP receptor lead to a failure of mammary development at the transition of the mammary bud into a phase of branching growth. As before, in the absence of the PTH/PTHrP receptor, the mammary epithelial cells degenerated and disappeared. These results established that it is amino-terminal PTHrP, signaling via the PTH/PTHrP receptor that is necessary for the initiation of branching morphogenesis in the mammary epithelium at E16.

Once we had established that amino-terminal PTHrP acting via the PTH/PTHrP receptor was responsible for PTHrP's effects on embryonic mammary gland development, we wanted to establish which cells express the PTHrP and PTH/PTHrP receptor genes, in order to begin to understand the mechanisms underlying the failure of mammary development in these knockout mice. Therefore we examined PTHrP and PTH/PTHrP mRNA expression during the embryonic mammary development of normal, BalbC mice by in situ hybridization histochemistry. We examined mammary development from E12 through birth. These experiments demonstrated that the PTHrP gene is highly and specifically expressed in mammary epithelial cells beginning at the mammary bud stage and continuing throughout embryonic development (see Fig. 4). In contrast, the PTH/PTHrP receptor is expressed specifically in the mesenchyme, both adjacent to the epidermis and surrounding the mammary epithelial bud (Fig. 4).

Summarizing these data, we have learned that PTHrP is necessary for the normal development and survival of the embryonic mammary epithelium. It appears that PTHrP is produced by the mammary epithelium and signals to the mammary mesenchyme. In the absence of this signal, the mammary mesenchyme cannot support morphogenesis of the mammary epithelium past the bud stage. In the absence of PTHrP signaling, the mammary bud fails to elongate and initiate the process of branching growth necessary to form the neonatal mammary gland. Instead, the mammary epithelium degenerates and disappears. Our current working hypothesis is that PTHrP's effects on the mesenchyme are critical for the initiation of branching morphogenesis in the mammary gland during embryonic development. We are currently attempting to characterize PTHrP's effects on mammary mesenchymal cells in the hopes of elucidating the mesenchymal factor or factors downstream of PTHrP that are responsible for the initiation of branching morphogenesis in the embryonic mammary epithelium.

These experiments have progressed nicely this past year, and are generally on time with regards to the original Statement of Work. Given our unexpected findings, we performed many experiments that were not part of the original proposal but that were necessary to characterize fully the effects of the lack of PTHrP on embryonic mammary development. We are currently initiating several experiments to attempt to examine the effects of the loss of PTHrP during later stages of mammary development. We are transplanting the mammary buds from E13 - E15 PTHrP- and PTH/PTHrP receptor-knockout embryos into the cleared fat pads of 4-week old nude mice. If these mammary buds grow within the host fat pad they will offer us the chance to examine adolescent and pregnant mammary development in the absence of PTHrP and its receptor.

Technical Objective 2. - The effects of PTHrP on the branching morphogenesis of mammary epithelial cells.

We have initiated the studies in this specific aim by characterizing our primary cultures of mammary stromal cells with regards to their cellular composition and their responses to PTHrP. Using a series of antibodies to keratins 8 and 18 (specific to luminal epithelial cells), keratin 14 (present in myoepithelial and some luminal epithelial cells) and vimentin (present in stromal cells) we have established, by immunocytochemistry, that our stromal cell primary cultures are composed of approximately 90 -95% stromal cells and 5 - 10% myoepithelial cells. We have demonstrated that these cells do not produce PTHrP, but contain the PTH/PTHrP receptor, and thus appear to be one "PTHrP-target" cell population in the mammary gland. Using these cultures we have established that mammary stromal cells respond with a 3 fold increase in cAMP which peaks in 2 minutes and begins to decline at 10 minutes. We have begun to examine the effects of PTHrP on the ability of these stromal cells to support branching morphogenesis by comparing the growth of epithelial cells cultured from the K14-PTHrP transgenic mice as compared to normal cells in collagen gel co-cultures (see original proposal). Preliminary experiments have demonstrated that the overexpression of PTHrP in these epithelial cells results in an impairment of branching morphogenesis as demonstrated by a 60-70% reduction in both the size and branching complexity of the epithelial colonies (see Fig. 5).

Given the additional experiments necessary to characterize the phenotype described in the first technical objective, we are a little behind the original timeline for this technical objective. However, we have initiated all the experiments planned for the first year, and I am confident that we will make rapid progress as these experiments will be a major focus of the second year.

Technical Objective 3. - The effects of PTHrP on estrogen- and progesterone-stimulated proliferation of mammary epithelial cells.

We have not yet initiated these experiments. As outlined in the Statement of Work, we plan to begin these experiments in year two of the proposal.

Technical Objective 4. - The effects of PTHrP on mammary tumor formation in GR mice.

Given the extra effort that was required to characterize the PTHrP-knockout phenotype (as described above in Section 1), we are somewhat behind schedule with this specific aim. We have just recently finished generating and identifying our cohort of GR/PTHrP and GR/nl female mice, and are now in the process of putting these mice through multiple pregnancies. As expected, there have been no tumors that have arisen in virgin mice. In

addition, based on several mice that were sacrificed after one pregnancy, it appears that PTHrP overexpression does not prevent the formation of hyperplastic alveolar nodules. However, these results are preliminary and we await the completion of the entire study in order to determine if PTHrP can influence the formation of MMTV-associated mammary tumors.

CONCLUSIONS

Our major findings in the first year of this project have been that, in the absence of PTHrP, mice have no mammary glands. It is clear that PTHrP is absolutely required for the initiation of branching morphogenesis and for mammary epithelial cell survival during embryogenesis. At this stage, PTHrP appears to act in a paracrine fashion on mammary stromal cells, for the PTH/PTHrP receptor gene is expressed exclusively in the embryonic mesenchyme and mice with a disrupted PTH/PTHrP receptor gene also manifest a failure of mammary epithelial development. We think that these are truly exciting findings. Combined with our previous results with PTHrP overexpression in the mammary gland, these results demonstrate that PTHrP is important in regulating the epithelial-mesenchymal communications that govern mammary ductal proliferation throughout the life-cycle of the mammary gland. It is interesting that in the absence of PTHrP, the embryonic stroma appears to loose not only the ability to support morphogenesis but also the ability to support the continuing survival of the mammary epithelial cells. It is known that stroma is often an important contributor to the transformed phenotype (13), and we are excited at the possibility that the effects of PTHrP on mammary stromal cells might influence the growth and/or survival of breast carcinoma cells. Therefore, as we continue in this project over the coming year, we will pay particular attention to defining the effect of PTHrP on stromal cells and how these impact on the proliferation and morphogenesis of mammary epithelial cells.

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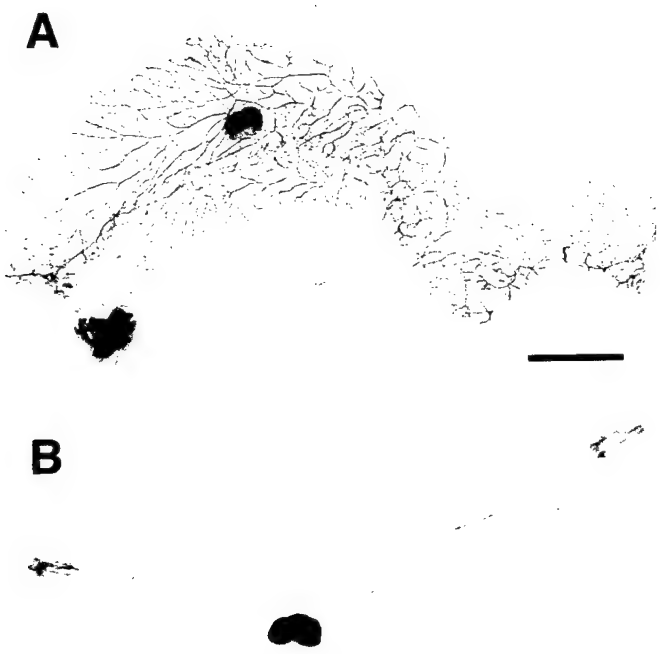

Figure 1. Whole mounts of the number 4, inguinal mammary glands from four month-old female mice were prepared by resection in toto, fixation in acid-ethanol and staining in carmine red. The normal gland (A) is characterized by a fully branched epithelial duct system surrounding the central lymph node. In the rescued-knockout or Col II - PTHrP/PTHrP - null gland (B), however, epithelial structures are completely absent; only the lymph node and the vasculature are present in the fat pad. The scale bar represents 5 mm.

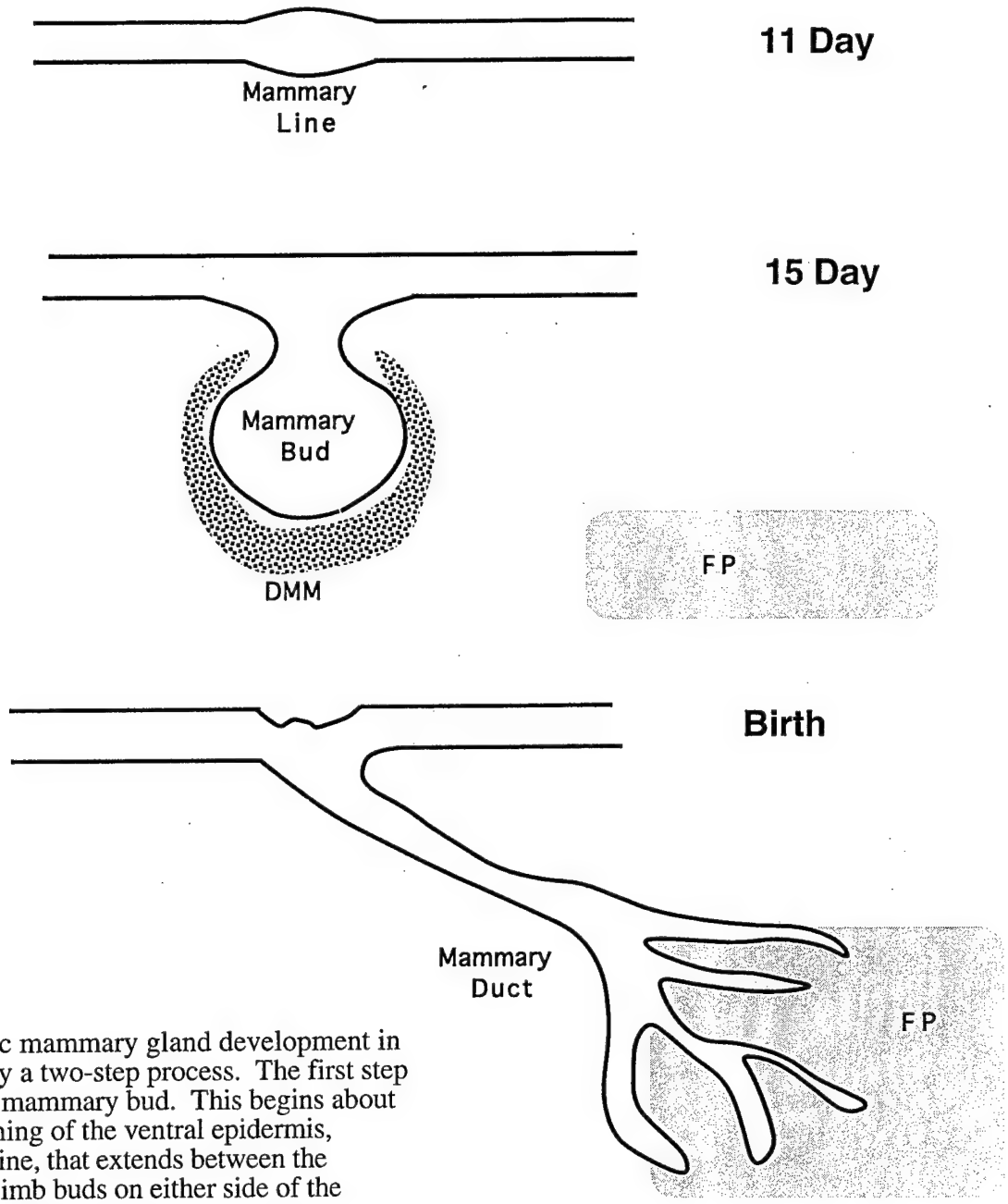

Figure 2. Embryonic mammary gland development in the mouse is essentially a two-step process. The first step is the formation of the mammary bud. This begins about E10 - E11, as a thickening of the ventral epidermis, termed the mammary line, that extends between the anterior and posterior limb buds on either side of the ventral surface of the embryo. By E12 - E13, the mammary lines have given rise to 10 distinct bud-like structures composed of an invagination of epithelium enveloped by a condensation of stroma known as the dense mammary mesenchyme (DMM). The precursor of the fatty stroma into which the gland will grow, the mammary fat pad (FP), forms separately beneath the dermis. This first (mammary bud) stage persists until E16 when the second phase, that of branching growth, begins. This consists of a growth spurt that results in the elongation of the mammary rudiment, its penetration into the developing mammary fat pad, and the initiation of branching morphogenesis. The end result is the transformation of the mammary bud into a branched tube that forms the sexually immature mammary gland present at birth.

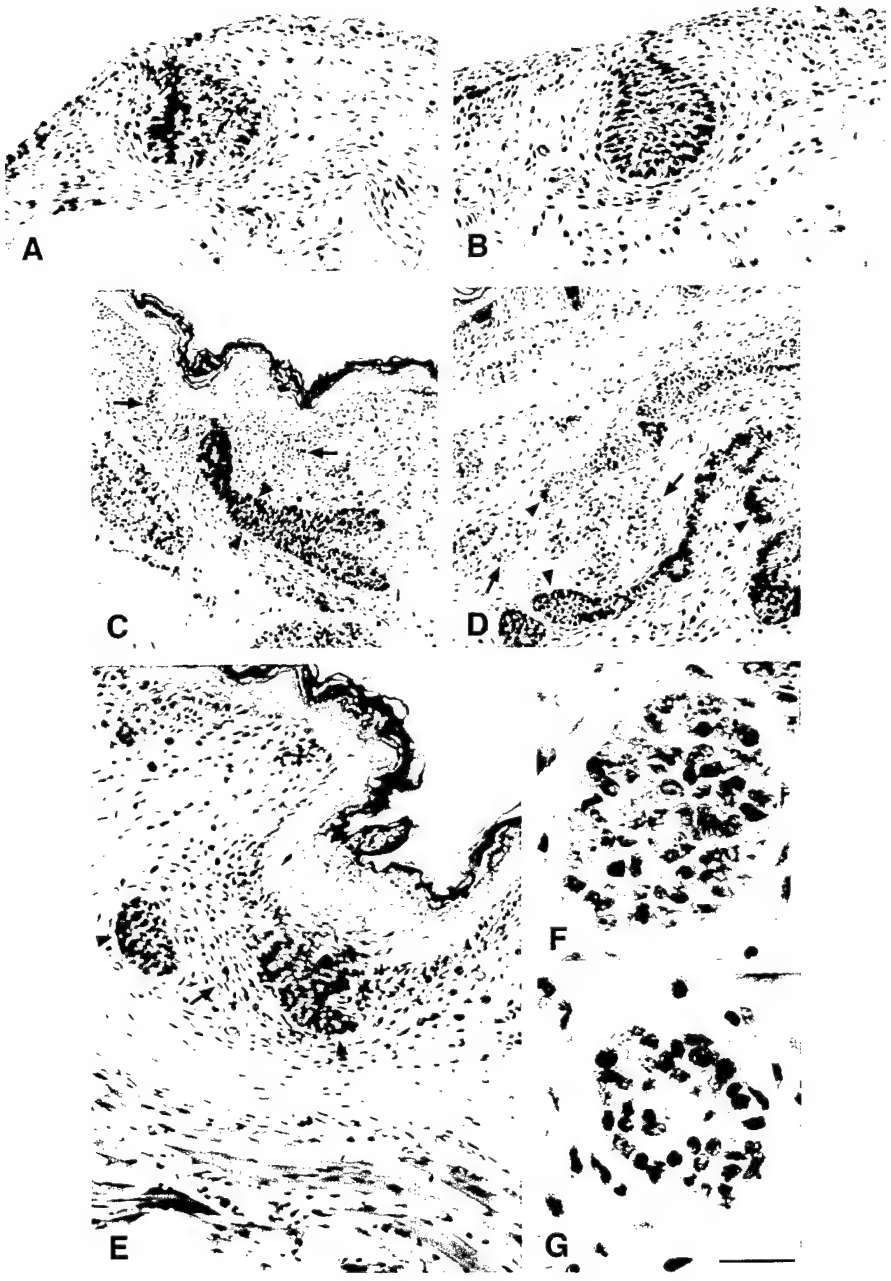

Figure 3. A & B are photomicrographs of H&E-stained sections through mammary buds dissected from a normal littermate (A) and a PTHrP-knockout (B) embryo at E15. At this stage, the microscopic appearence of the PTHrP-knockout buds was entirely normal. C through G are photomicrographs of H&E-stained sections through mammary glands dissected from PTHrP-knockout (E & G) and normal littermate (C,D & F) embryos at E18. In a normal embryo (C & D) one can see the primary epithelial duct (arrowhead in C) arising from the epidermis and extending below the dermis where it branches (arrowheads in D) and makes contact with the preadipocytes (arrows in D) within the developing fat pad. In contrast, in the PTHrP-knockout embryos (E) the epithelial duct (arrowheads) does not extend out of the upper regions of the dermis and becomes surrounded by an abnormally dense condensation of fibroconnective tissue (arrow in E). F & G are high power photomicrographs of mammary epithelial ducts in crossection taken from a normal (F) and PTHrP-knockout (G) embryo at E18. Note that in the knockout duct (G) the epithelial cells appear to be degenerating; many nuclei are pycnotic, the cell cytoplasm appears reduced and somewhat vacuolated and the cells are seperating from the basement membrane. Scale bar represents 16 microns in A&B, 25 microns in C&D, 17 microns in E and 5 microns in F&G.

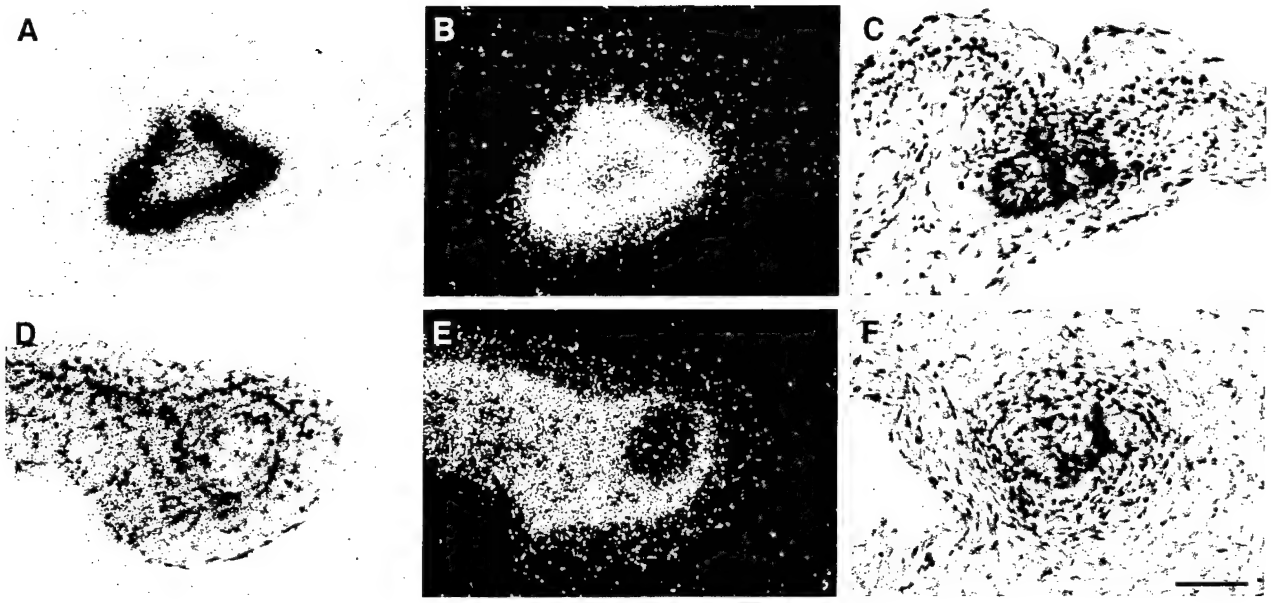

Figure 4. Localization of PTHrP and PTH/PTHrP mRNA expression in normal embryonic mammary glands. A-C. In situ hybridization for PTHrP mRNA in normal mammary rudiments at E16. A & B are brightfield and darkfield images, respectively, of the same section hybridized with antisense probe. C is a brightfield image of a similar section hybridized to sense probe as a control. Note that PTHrP mRNA is found in the mammary epithelial cells, especially those located peripherally. There is no hybridization within the mesenchyme. Note the lack of hybridization of the sense probe (compare A & C). D-F. In situ hybridization for PTH/PTHrP receptor mRNA in normal mammary rudiments at E15. D & E are brightfield and darkfield images, respectively, of the same section hybridized with antisense probe. Note that PTH/PTHrP receptor mRNA is found within the dense mammary and dermal mesenchyme; there is no receptor mRNA expressed within the mammary epithelial cells. F is a brightfield image of a similar section hybridized to PTH/PTHrP receptor sense probe as a control. Note the lack of signal as compared to D. Scale bar represents 15 microns for all panels.

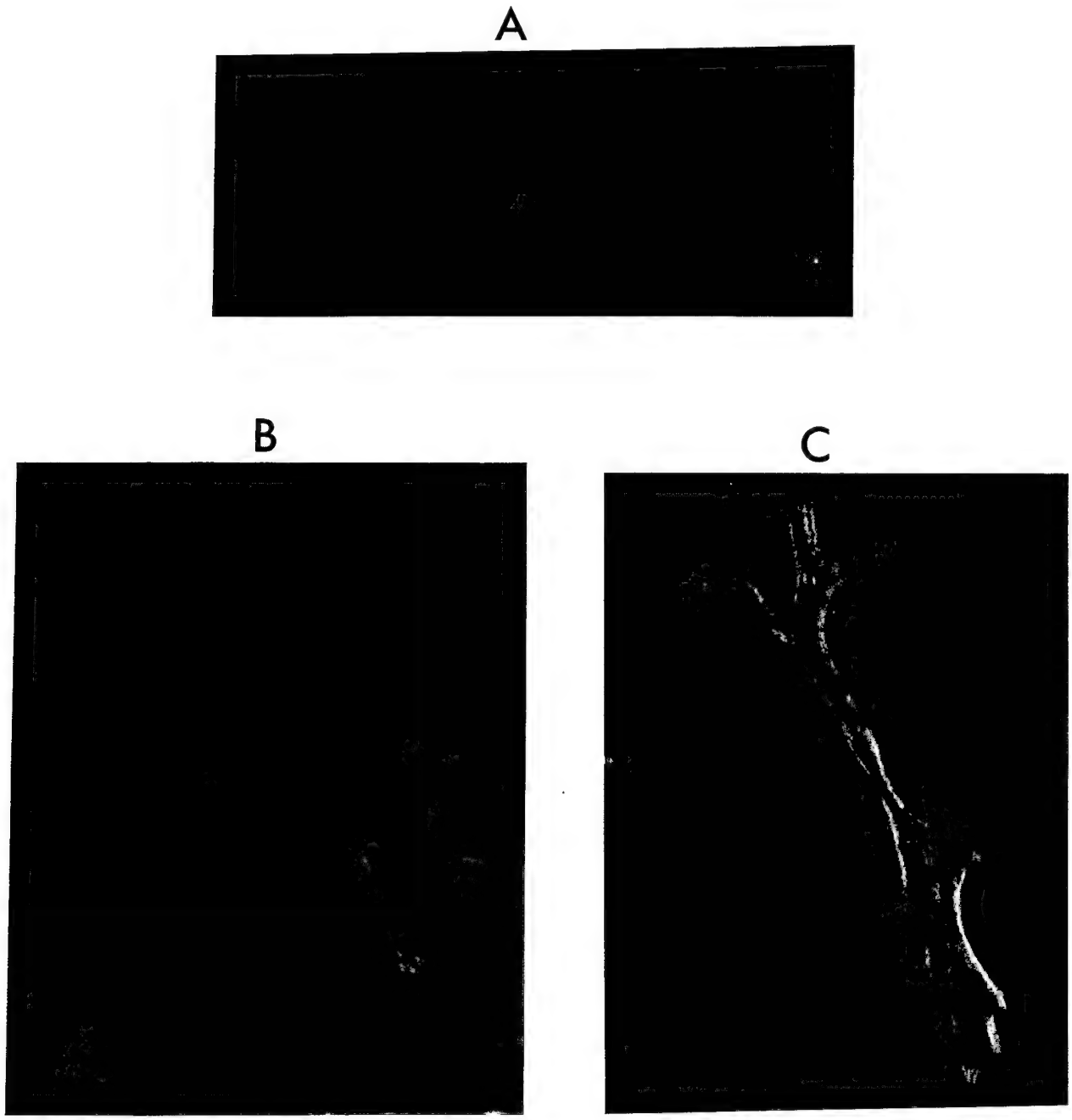

Figure 5. Comparison of colonies derived from normal and PTHrP-overexpressing mammary epithelial cells co-cultured with mammary stromal cells in Type-1 collagen gels. In the absence of stromal cells the mammary epithelial cells grow as round colonies (A). In the presence of mammary stromal cells, epithelial cells from normal mice form branched, tubular colonies that grow out and interconnect (B). Epithelial cells, derived from K14-PTHrP transgenic mice, that overexpress PTHrP (C) also form branched colonies in collagen gel co-cultures, but the overall size and degree of branching of the colonies is reduced.

Rescue Of The Parathyroid Hormone-related Protein Knockout Mouse Reveals Multiple Extraskeletal Developmental Abnormalities

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Key Words: epithelial-mesenchymal interactions, branching morphogenesis, gene targeting, transgenic mice, keratin 14, collagen II, tooth eruption, epidermis, mammary gland, differentiation

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SUMMARY

Mice with a homozygous deletion of the parathyroid hormone-related protein (PTHrP) gene die at birth with a chondrodystrophic phenotype characterized by premature chondrocyte differentiation and accelerated bone formation, while overexpression of PTHrP in the chondrocytes of transgenic mice via the procollagen II promoter results in the delay of chondrocyte maturation and endochondral ossification. Replacement of PTHrP expression in the chondrocytes of PTHrP-knockout mice using the procollagen II-driven transgene effects the correction of the lethal skeletal abnormalities and generates animals which are effectively PTHrP-null in all sites other than cartilage. These rescued PTHrP-knockout mice survive to at least six months of age but display a number of developmental defects, including abnormal epidermal differentiation, the absence of mammary epithelium, and the failure of tooth eruption. The further introduction of a PTHrP transgene targeted to the epidermis and mammary gland by virtue of the keratin 14 promoter results in the restoration of the normal programs of development in these organs. Since keratin 14 is also expressed by the enamel epithelia, the expression of the PTHrP transgene at this site in doubly transgenic animals corrects the defect in tooth eruption as well. PTHrP thus represents an essential signal in the epithelial-mesenchymal interactions involved in the later stages of development of the epidermis, mammary gland and tooth.

INTRODUCTION

Although parathyroid hormone-related protein (PTHrP) was originally discovered by virtue of its elaboration from tumors associated with the syndrome of humoral hypercalcemia of malignancy, it has become increasingly clear that this is the only circumstance in which sufficient quantities of the peptide are released into the general circulation to exert systemic effects (Nissenson and Strewler, 1992; Broadus and Stewart, 1994; Martin and Moseley, 1995; Philbrick et al., 1996). The PTH and PTHrP genes represent the present sequelae of an ancient gene duplication; structural similarity and sequence homology at the amino termini of the respective mature peptides mediate their binding to and stimulation of the type I PTH/PTHrP receptor with equal affinity (Jüppner et al., 1991; Abou-Samra et al., 1992). Activation of this G protein-coupled receptor on osteoblasts and renal tubule cells has been shown to be responsible for the classical effects of PTH on calcium and phosphate metabolism and, similarly, to mediate the paraneoplastic effects of PTHrP. Localization studies in fetal tissues by immunohistochemistry and in situ hybridization have shown PTHrP and its mRNA to be typically expressed in a focal pattern within surface epithelia, while the type I receptor is diffusely distributed in the adjacent mesenchyme (Campos et al., 1991; Lee et al., 1995). In skeletal growth plates, PTHrP is expressed in the perichondrium and maturing chondrocytes, while its receptor is primarily found in the proximal prehypertrophic layer. Thus, PTHrP appears to act locally in a paracrine or autocrine fashion. Segregation of the PTH and PTHrP signaling pathways is likely accomplished through a number of mechanisms, one of which appears to be the generation of high local concentrations of PTHrP and its lability or sequestration in the microenvironment surrounding sites of expression. In addition, the density of type I receptors is high on PTH target cells in bone and kidney, but relatively low in most of the presumed PTHrP target tissues, which are unresponsive to circulating levels of PTH (Nissenson and Strewler, 1992; Lee et al., 1995; Philbrick et al., 1996).

Over the past several years, a number of experiments with transgenic mice have implicated PTHrP as a developmental regulatory molecule. Targeted overexpression of PTHrP in the

epidermis produced both delay and failure of hair follicle initiation (Wysolmerski et al., 1994), and overexpression in mammary myoepithelial cells both delayed and profoundly diminished the extent of ductal proliferation and branching morphogenesis (Wysomerski et al. 1995). Disruption of the PTHrP gene in knockout mice resulted in a lethal skeletal dysplasia characterized by the premature maturation of chondrocytes and accelerated mineralization of bone (Karaplis et al., 1994), while overexpression of the peptide in the prehypertrophic chondrocytes of transgenic mice caused a profound delay in chondrocyte differentiation and endochondral ossification (Weir et al., 1996). In addition, a constitutively-activated mutant type I receptor has been found to be the cause of Jansen-type metaphyseal chondrodysplasia in humans, a disease characterized by delayed endochondral bone formation (Schipani et al., 1995). PTHrP thus appears to function as an attenuator of programmed differentiation in the genesis of a number of organ systems.

Since the perinatal lethality in the PTHrP-knockout mice has prevented analysis of any potential consequences of the absence of the peptide on the postnatal development of organs such as the skin and the mammary gland, we attempted to correct the primary skeletal defects in the knockouts by specifically replacing PTHrP expression in the skeleton with a chondrocyte-targeted transgene. As described in the present manuscript, rescued PTHrP-knockout animals display defects in epidermal differentiation, an absence of mammary epithelium and a failure of tooth eruption. Further, all three of these extraskeletal defects can be remediated by the specific replacement of PTHrP in the affected sites.

MATERIALS AND METHODS

Breeding and maintenance of mouse lines

CD-1 outbred mice were purchased from Charles River Laboratories (Wilmington, MA) and served as breeding partners for the maintenance of all mouse lines. The generation of PTHrP-knockout mice and of procollagen II-PTHrP and keratin 14-PTHrP transgenic mice has been described (Karaplis et al., 1994;

Wysolmerski et al., 1994). Rescued PTHrP-knockout animals were fed a liquid diet containing 1000 calories per ml and composed of approximately 63.2% carbohydrate, 20.2% protein, 5.4% fat, 4.0% fiber, 3.2% ash and 4.0% moisture (Bio Serv, Frenchtown, NJ). All other animals were fed a standard diet of mouse chow and water. Genotyping was carried out by PCR amplification of tail biopsy homogenates and employed the following primer sets: human growth hormone transgene tag (171 bp product, Weir et al., 1996), murine glyceraldehyde phosphate dehydrogenase (259 bp product, Weir et al., 1996), human keratin 14 promoter region (approximately 500 bp product, 5'-CACGATACACCTGACTAGCTGGGTG forward, 5'-CATCACCCACAGGCTAGCGCCAACT reverse), murine procollagen II promoter/human PTHrP cDNA junction segment (approximately 510 bp product, 5'-TCTTAGCATTCTTGGAGAAC forward, 5'-ATCAGATGGTGAAGGAAG reverse), wild-type murine PTHrP gene (421 bp product, 5'-GCTACTGCATGACAAGGGCAAGTCC forward, 5'-GAGCCCTGCTGAACACAGTGAACAG reverse), bacterial neomycin gene (315 bp product, 5'-GGAGAGGCTATTCGGCTATGAC forward, 5'-CGCATTGCATCAGCCATGATGG reverse). Due to their apparent hypothermia, rescued knockout mice were housed on a heating pad set at 44°C. These animals were also groomed weekly to clean matted fur, remove adhered diet and prevent mucus accumulation around the eyes. Hypogonadal females were treated with 8 IU of pregnant mares' serum (a source of follicle stimulating hormone, FSH), followed 48 hours later by 8 IU of human chorionic gonadotropin (HCG, as a substitute for luteinizing hormone, LH) and the reproductive organs were examined after 24 hours (Hogan et al., 1994).

Tissue analysis and histology

Dissected soft tissues were fixed either in 10% buffered formalin or in freshly prepared 4% paraformaldehyde in 1X PBS, dehydrated and embedded in paraffin. Typically, sections were cut at 5 μm and stained with hematoxylin and eosin (H and E). Full thickness skin samples were taken from the mid-dorsum of six- to eight-week-old female mice. Mammary glands were resected, fixed in acid alcohol, stained with carmine aluminum and cleared of fat to prepare whole mounts as described (Wysomerski et al. 1995). Neonatal skeletons were stained with alizarin red S to differentiate

calcified tissues (Weir et al., 1996). For non-decalcified histology of neonatal teeth, mandibles were dissected free of soft tissue, fixed in 40% ethanol, dehydrated with graded ethanols and toluene, embedded in methyl methacrylate, sectioned at 4 μm and stained with toluidine blue (Baron et al., 1983). Mandibles from one-week-old mice were fixed in formalin, decalcified in 10% EDTA for ten days and then processed in the same manner as soft tissues. Radiographic analyses of tooth development were carried out with a Hewlett-Packard Faxitron at 27 kV for 40 seconds. For detection of tartrate-resistant acid phosphatase (TRAP) activity, neonatal mandibles were fixed and decalcified as above, and then embedded in glycol methacrylate, sectioned and stained with naphthol AS-BI phosphate and diazotized fast garnet GBC salt (Sigma, St. Louis, MO) in the presence of tartrate, followed by counterstaining with Grocott's light green. Mouse skulls were prepared by decapitation, dissection of skin and muscle, removal of brain tissue through the foramen magnum and the elimination of the remaining soft tissue by incubation with Dermestid beetle larvae (Hefti et al., 1980, graciously provided by F. Sibley at the Yale Peabody Museum) for several days at 37°C. Keratin 14 expression was localized with an affinity-purified rabbit polyclonal antibody (provided by D. Roop, Houston, TX) at a dilution of 1:500, biotin-conjugated secondary antibody and an avidin-peroxidase detection system (Vector Laboratories, Burlingame, CA) using diaminobenzidine as a chromogen.

Results

Generation of rescued PTHrP-knockout mice.

The primary abnormality displayed by the PTHrP-knockout mouse is a defect in skeletal development which is characterized by accelerated ossification and resultant foreshortening of the endochondral bones of the axial and appendicular skeleton (Karaplis et al., 1994),. The death of these mice shortly after birth appears to be due to inappropriate ossification of the costal cartilage and subsequent respiratory failure. Most other tissues and organs appear to have developed normally, but the neonatal death of these animals has prevented a proper evaluation of function in vivo. We therefore attempted to rescue the PTHrP-knockout mouse by specifically

replacing the peptide in endochondral bone. This approach was designed to create an animal which was effectively PTHrP-null in all tissues except bone, thereby allowing the assessment of potential effects of the absence of PTHrP in developmental programs which occur predominantly postnatally, such as the initiation of hair follicles and branching morphogenesis in the mammary gland.

To this end, the disrupted PTHrP gene (null allele) was first crossed onto a CD-1 outbred background for several generations to minimize any potential exacerbating effects of the original inbred background. Next, we took advantage of a transgenic model of PTHrP overexpression targeted to chondrocytes via the murine procollagen (α1) type II promoter (Weir et al., 1996). However, rather than using a line of transgenic mice with high levels of PTHrP expression in bone associated with a profound delay in endochondral ossification and short-limbed dwarfism, we chose instead a line with lower expression levels, in which retarded mineralization can be detected only in the tail vertebrae at birth and normalizes soon thereafter (see Fig. 2). These col II-PTHrP transgenics, derived following extensive interbreeding with CD-1 outbred mice, were crossed to PTHrP-null heterozygotes, and resultant offspring carrying both the transgene and a PTHrP null allele (occurring at a ratio of one in four) were again crossed to PTHrP-null heterozygotes, thereby generating col II-PTHrP transgenic, PTHrP-null homozygous mice or "rescued knockouts".

Gross phenotype

Rescued PTHrP-knockout mice were born at the appropriate Mendelian ratio of one in eight, but less than half survived past three weeks of age. Although indistinguishable from their littermates at birth, these animals failed to thrive and exhibited a 50% reduction in growth rates by the time of weaning. As a result, adult mice were symmetrically dwarfed by approximately one-half with respect to both size and weight. Due to an apparent lack of functional dentition, rescued animals were unable to eat solid food and were therefore placed on a liquid diet available ad libitum at the time of weaning. Since the maintenance energy requirements of the rescued knockout mice were calculated to be roughly 500 cal/g per day (160 kcal/kg0.75 body weight) and daily calorie consumption would be

expected to be almost twice that amount (Bernier et al., 1986), the measured metabolizable energy intake of approximately 600 cal/g per day was likely insufficient to sustain additional growth in these animals. This deficient nutritional intake was not due to impaired dentition; both normal littermates and other toothless mouse mutants (eg., op/op and c-src null) exhibited rapid weight gain on this liquid diet (unpublished observations). Adult mice were lethargic and led a largely sedentary existence. Respiration rates were within the normal range, but breathing was clearly labored; this may have been due to a failure of the collagen II-driven PTHrP expression to restore optimal flexibility to the rib cage. Although the attrition rate lessened considerably after successful weaning, adult mice progressively weakened and died; thus far, none has survived beyond six months. Other distinguishing features included a matted, oily coat, abnormally long nails, and a foreshortened snout and domed calvarium typical of a classical cranial chondrodystrophy (Johnson et al., 1993, Fig. 1). Resultant shallow eye sockets were the apparent cause of the noticeable ocular proptosis and attendant secretions.

Routine anatomical and histological examination of a number of internal organs and tissues from these mice revealed no readily discernible abnormalities. These included heart, lungs, liver, kidneys, pancreas, brain, salivary glands, skeletal muscle, and smooth muscle from the gastrointestinal tract and bladder. Organs were typically half the size of littermate controls and often contained fewer cells and less interstitium, but otherwise appeared to be structurally and developmentally appropriate for the age of the mouse. The absence of PTHrP gene expression in extraskeletal tissues was confirmed by RNase protection with a murine riboprobe (Weir et al., 1996, data not shown). Spleen, thymus and lymph nodes were only one quarter of the normal size and contained proportionately fewer cells, but analysis by fluorescence activated cell sorting (FACS) did not detect any significant shifts in critical cell populations (markers included those for B and T cells, CD4 and CD8 T cells, and $\alpha\beta$ and $\gamma\delta$ T cells, among others). Blood white cell counts were also low (again approximately 25% of normal), but based on infection rates and wound healing, there was no evidence of immune dysfunction in these animals. Despite the ability of PTHrP to augment islet mass in an overexpression model, no morphological differences could be

detected in pancreata from rescued knockouts and blood glucose levels were within the normal range. As expected, serum levels of calcium and creatinine were normal, indicating that calcium homeostasis and renal function were unperturbed by the absence of PTHrP. Reproductive organs appeared unremarkable until puberty, at which time a developmental delay became apparent; male testes, although often oversized, displayed a deficiency of Leydig cells and fully differentiated sperm, while mature follicles and corpora lutea were rare in the ovaries of females. Other components of the reproductive systems in the male (prostate, preputial gland and seminal vesicle) and in the female (oviduct and uterus) were also found to be histologically normal but immature. Consistent with these findings were the observed passivity of the males, the indeterminate appearance of external genitalia in prepubertal animals and the lack of mating behavior by either sex. This hypogonadism probably does not represent a direct effect of the absence of PTHrP but rather may be due to the meager stores of body fat in these animals (Chehab et al., 1997). Administration of FSH and HCG to adult rescued females induced both follicle maturation and ovulation, indicating full ovarian competence. Pituitaries were histologically normal and contained immunoreactive FSH and LH (not shown).

Correction of the primary skeletal defects

As shown in Fig. 2, differential staining of calcified tissue in neonatal rescued mice indicated substantial correction of the skeletal abnormalities seen in the PTHrP-knockouts. Ossification of long bones, pelvis and vertebrae was not premature, and there was no evidence of inappropriate mineralization of the costal or intersternebral cartilages. Endochondral growth plates also appeared to be essentially normalized (not shown). Thus, the targeting of PTHrP expression via the procollagen II promoter to the prehypertrophic chondrocytes that bear the type I PTH/PTHrP receptor appeared sufficient to restore the normal program of differentiation in endochondral bone. This approach did not, however, ameliorate the cranial chondrodysplasia seen in the knockout mice. Rescued animals displayed a reduction in the length of the maxillary-occipital axis and doming of the calvarium. Although the procollagen II gene has been shown to be expressed in both the

chondrocranium and at some sites of intramembranous bone formation (Sandell et al., 1994), it is apparent that the transgenic expression of PTHrP is either temporally inappropriate or quantitatively insufficient to functionally restore normal craniofacial bone development.

Findings in skin and mammary gland

Our previous studies targeting overexpression of PTHrP in the epidermal and mammary epithelium suggested that this peptide functions as an attenuator of programmed differentiation in these tissues (Wysolmerski et al., 1994; Wysolmerski et al., 1995). Since the targeted overexpression of PTHrP in bone and its ablation in the knockout mouse produce reciprocal effects, we examined the skin and mammary gland of rescued PTHrP-knockout mice for potential consequences of the peptide's absence.

In skin, several major alterations were apparent (Fig. 3). First, the stratified epithelium in the epidermis was reduced to a single layer of undersized keratinocytes which had a flattened, squamous appearance as opposed to the more cuboidal shape typical of murine epidermal basal cells. This was accompanied by multiple sheets of overlying, keratinized material representative of extreme hyperkeratosis. Second, hair follicles appeared to be inappropriately keratinized, and the animals manifested progressive hair loss with age. Third, there was a striking reduction in both the size of sebaceous glands present in the dermis and in the number of cells within each gland. An increase in the rate of sebaceous cell maturation and holocrine rupture could account for both the decreased cellularity of the gland and the accumulation of oily secretions on the coats of these mice. Taken together, these findings are consistent with an acceleration of the differentiation program in the epidermis and epidermal appendages (Kopan and Fuchs, 1989; Eckert et al., 1997). Other findings in skin included a fibrotic and cellular dermis and a marked diminution in subcutaneous fat. Body fat in general was scarce in these animals, but small deposits of interscapular brown fat and mesenteric white fat could be identified, suggesting that the relative abundance of adipose tissue may simply reflect the compromised nutritional state of these animals.

The growth and development of ductular epithelium in the murine mammary gland occurs in three distinct stages. During embryogenesis, from approximately E16 until birth, an epidermally-derived mammary rudiment penetrates the adjacent fat pad and forms a limited ductal tree. Subsequent growth over the next three weeks is isometric, rather than allometric, and simply keeps pace with the overall growth of the animal (Sakakura, 1987). In response to systemic hormones (principally estrogen and progesterone) during the period of sexual maturation from four to six weeks, ductal growth is extended to the borders of the fat pad and undergoes extensive ramification. Finally, during pregnancy and lactation, the ductal epithelium is induced to create additional side branches and the duct termini then differentiate into secretory alveoli, which expand to fill the entire fat pad (Daniel and Silberstein, 1987).

Unexpectedly, examination of the mammary glands from adult female rescued knockout mice revealed not accelerated differentiation as anticipated, but rather a total absence of ductal epithelium within the fat pads. As seen in Fig. 4, whole-mount analysis of the number 4 inguinal gland showed no evidence of either a branching network or a primary duct. Nipple structures in the epidermis also appeared to be lacking, suggesting either failure or early arrest in the morphogenesis of the organ. As described in the accompanying paper by Wysolmerski et al., (1997) embryonic mammary buds form appropriately, but fail to elongate and penetrate the fat pad and thus cannot initiate branching growth. Subsequent degeneration of the stunted primary duct eliminates the remaining mammary epithelium prior to birth. The expression of PTHrP thus appears to be critical for early mammary morphogenesis.

Failure of tooth eruption

The apparent absence of functional dentition in the rescued PTHrP-knockouts was entirely unanticipated. It was initially unclear whether this represented a failure of tooth morphogenesis or of tooth eruption. Dissection of the oral epithelium failed to uncover any evidence of incipient eruption of either molars or incisors. In order to examine the bone surfaces at expected sites of tooth eruption, skulls from adult rescued knockout mice and normal littermates were subjected to digestion by Dermestid beetle larvae until free of soft

tissue. As shown if Fig. 5, the cranial phenotype of the rescued PTHrP-knockout mouse consisted of a total absence of dentition superimposed on the classical features of a cranial chondrodystrophy. External incisors and molars were absent from both the mandible and maxilla, and the bone surfaces overlying expected sites of eruption were intact. Murine incisors normally emerge at approximately one week of age (Hay, 1961), followed by the molars (M1 through M3) beginning at two weeks (Cohn, 1957). Serial radiographs (Fig. 6) revealed a failure of tooth eruption in the rescued knockouts, and intensifying in the jaws of the mice indicated that the incisors and molars had become trapped and progressively impacted by the surrounding alveolar bone. Histologic examination of non-decalcified sections of neonatal mandibular incisors from rescued knockouts and normal littermates indicated that tooth morphogenesis was unaffected by the absence of PTHrP; rescued knockout incisors were fully developed and possessed mature layers of dentin and enamel (Fig.7). Unimpeded growth of alveolar bone in the rescued animal, however, compressed the epithelial layers surrounding the tooth to such a degree that the normally prominent ameloblast layer, which is responsible for enamel deposition, was destroyed. Inspection of decalcified molars at one week showed this same process of impaction occurring in these teeth prior to their scheduled eruption (Fig. 7). The ameloblast layer was still intact at this point, but the molar crypt had become choked with bone and the teeth were becoming increasingly distorted. Ultimately, the teeth become irreversibly ankylosed due to the fusion of the dental cementum with the encroaching alveolar bone.

The findings described above correspond to those classically associated with oseopetrosis, a disorder of impaired osteoclast function. The rate-limiting step in the process of tooth eruption is the resorption of bone overlying the crown to create an eruption pathway (Marks, 1995; Marks and Schroeder, 1996). Prior to the scheduled eruption of the first molars in the rodent at two weeks of age, there is an influx of mononuclear stem cells into the dental follicle and a concomitant increase in the number of multinucleated osteoclasts in the coronal portion of the bony crypt, which peaks at three days postnatally (Wise and Lin, 1995). Lesions in any of the principal stages of osteoclast development can disable the bone resorption process (Baron et al., 1993; Suda and Martin, 1995). For

example, teeth fail to erupt in three of the murine models of osteopetrosis, the op/op mutant and the c-fos and c-src knockouts, due to defects in osteoclast formation, differentiation and activation, respectively (Yoshida et al., 1990; Grigoriadis et al., 1994; Lowe et al., 1993). In the rescued PTHrP-knockout animals, multinucleated osteoclasts along the alveolar bone surfaces surrounding the neonatal incisor were clearly visible by light microscopy (Fig. 7E) and the enzymatic detection of tartrate-resistant acid phosphatase (TRAP), a marker of osteoclast differentiation, revealed intensive staining immediately adjacent to both incisors and molars in the pre-eruptive phase of development at day 1 postnatally (Fig. 7F). These results implicate a defect in osteoclast activation and not formation or differentiation in the eruption failure of the rescued knockouts. PTHrP can be detected in the dental lamina of rats as early as day 15.5 of embryogenesis and is strongly expressed in the reduced enamel epithelium surrounding the crowns of erupting molars and in the overlying layers of connective tissue and oral epithelia at two and a half weeks postnatally (Beck et al., 1995). The type I receptor, on the other hand, is expressed in the developing dental mesenchyme by E15 and on neighboring osteoblasts during the formation of alveolar bone shortly thereafter (Lee et al., 1995). The loss of PTHrP in the rescued knockout animals, therefore, presumably disrupts a type I receptor-mediated paracrine signaling pathway essential for the osteoclast activation that ultimately allows tooth eruption to occur.

Replacement of PTHrP expression in skin and the mammary gland

The expression of PTHrP driven by the procollagen II promoter did not faithfully recapitulate the precise spatial, temporal and quantitative pattern of the endogenous PTHrP gene and yet proved capable of effecting substantial correction of the skeletal abnormalities present in the PTHrP-knockout. To see whether a similar approach could be applied in the extraskeletal differentiation programs that appear to be regulated by PTHrP, we turned to the keratin 14-PTHrP transgene, which had been previously used to target the peptide to endogenous sites of PTHrP expression in the epidermis and mammary gland (Wysolmerski et al., 1994; 1995). Breeding of the K14-PTHrP transgene onto a PTHrP-null background

was carried out in a manner analogous to that employed previously. K14-PTHrP transgenic, PTHrP-null heterozygotes were first derived and then bred to the col II-PTHrP transgenic, PTHrP-null heterozygotes to obtain K14-PTHrP/col II-PTHrP double transgenic, PTHrP-null homozygotes at a frequency of 1 in 16. As might have been anticipated, these doubly transgenic, rescued PTHrP-knockout mice ("doubly-rescued knockouts") were identical to their col II-PTHrP rescued counterparts with regard to growth rate, body size, respiratory pattern, life span, normalized skeletal phenotype and persistent cranial chondrodystrophy.

Immediately obvious, however, was an improvement in the appearance of the coat, which was composed of clean, discrete, erect hairs typical of a normal animal (Fig. 8). Histological examination of skin from adult doubly-rescued animals revealed substantial amelioration of the array of defects seen in the absence of PTHrP. As shown in Figure 8, the hyperkeratosis was remedied and the epidermal and dermal changes was largely normalized. Hair follicles were not inordinately keratinized, and there was no evidence of hair loss with age. Finally, sebaceous glands were no longer reduced in size or hypocellular, and normal levels of subcutaneous fat were restored. Interestingly, the overgrowth of nails was not corrected, presumably due to the failure of this promoter to effectively target the relevant site of PTHrP expression. Thus, expression of PTHrP in basal keratinocytes and outer root sheath cells under the direction of the heterologous K14 promoter appeared to be able to correct the majority of skin-related changes incurred in the PTHrP-null state.

In the mammary gland, as in skin, there is considerable overlap in the respective expression patterns of PTHrP and keratin 14 with regard to developmental timing and the cell-types involved Wysomerski, 1994, 1995). As a result, the transgenic expression of PTHrP was able to effect considerable restoration of the differentiation program in this site as well. Although the development of nipples was not evident in post-pubertal, doubly-transgenic rescued females, whole-mount analysis of mammary glands demonstrated the presence of incipient ductal growth in the fat pad (Fig. 9), indicating the successful initiation of branching morphogenesis. The growth of ductal epithelium in these mice did not, however, extend throughout the fat pad, as occurs in normal

females, and was likely compromised by the hypogonadal state of the animals. To address this issue, adult, doubly-rescued females were administered 17 β-estradiol (1mg/day) and progesterone (1ng/day) subcutaneously for two weeks, a regimen which induces full sexual differentiation of the mammary gland in ovariectomized, normal females (Wysolmerski et al., 1995). As shown in Fig. 9, ductal growth in the mammary glands of adult, doubly-rescued females treated with hormones had progressed to the limits of the fat pad and was appropriately branched. Therefore, the replacement of PTHrP expression in the developing mammary gland via the K14 promoter was sufficient to support the early morphogenesis of the ductal epithelium and to allow its subsequent growth and ramification.

Restoration of tooth eruption

Correction of the failure of tooth eruption in col II-rescued PTHrP-knockouts by the K14 transgene had not been anticipated, but the presence of externalized incisors in the doubly-rescued animals became readily apparent prior to weaning. Both incisors and molars were observed to erupt normally and on schedule. Several cytokeratins, including K14, have been reported to be expressed in the epithelial layers surrounding the developing tooth (Feghali-Assaly et al., 1994). We therefore confirmed the presence of K14 expression by immunohistochemistry in normal murine teeth prior to their eruption. As shown in Fig. 10, intense staining was visible throughout the layers of the reduced enamel epithelium of a neonatal incisor; molars exhibited similar staining (not shown). This expression pattern replicates that for PTHrP at this stage of development and thus provided the basis for the observed phenotypic rescue. Although aligned normally at the time of eruption, the lower incisors of adult double rescues displayed a striking overgrowth and increasing malformation. An unusual property of murine incisors, but not molars, is that they continue to grow throughout the animal's lifetime and are impeded only by occlusion with the opposing teeth (Burn-Murdock, 1971; Marks and Schroeder, 1996). As a result of the uncorrected chondrodystrophy in the skulls of doubly-rescued mice, the lower, mandibular incisors protrude beyond the upper incisors in the foreshortened maxilla. This is best appreciated in a sagittal view of a cranial radiograph as

shown in Fig. 10. The resultant malocclusion allows unchecked growth of the lower incisors, which then exhibit increasing deviation from the midline. Upper incisors remain partially occluded and were consequently less affected.

DISCUSSION

While the dysregulation of a gene of interest through targeted overexpression has often proven to be an extremely informative tool for unearthing potential function, the ablation of gene expression by homologous recombination remains, in most cases, the only means by which prima facie evidence for a unique and critical role in vivo can be obtained. In the case of PTHrP, overexpression models have implicated the peptide as an attenuator of programmed differentiation in the epidermis, mammary gland and endochondral bone (Wysolmerski et al., 1994, 1995; Weir et al., 1996), while disruption of the PTHrP gene has established a unique and critical role for the peptide in skeletal development (Karaplis et al., 1994). Similarly, the rescue of the PTHrP-knockout mouse has allowed us to assess the effects of the peptide's absence on the development and function of other organ systems in vivo. Given the array of tissues which express PTHrP and in which a specific function for the peptide might have been justifiably anticipated, it is noteworthy that the developmental abnormalities detected were quite discrete. For example, a number of organ systems which develop in whole or in part by branching morphogenesis, such as kidney, pancreas, lung, salivary gland and prostate (Birchmeier and Birchmeier, 1993), appeared unaffected by the lack of PTHrP. One explanation for this is, of course, redundancy of function, where two or more molecules serve the same or overlapping roles, while another is the sensitivity with which a given phenotype can be detected. For example, PTHrP has been shown to relax smooth muscle in the microvasculature, gastrointestinal tract, bladder, and myometrium (reviewed in Phibrick et al., 1996), yet without highly accurate respective assessments of blood pressure, motility, distention, and the timing of parturition, potential consequences of its absence in these sites could not be appreciated.

Rescued PTHrP-knockout animals displayed three major developmental abnormalities: an acceleration of differentiation in the

epidermis and epidermal appendages, the absence of mammary ductal epithelium, and the failure of tooth eruption. The initial step in the morphogenesis of all three of these systems is an invagination of surface ectoderm in response to a primary mesenchymal condensation and, in each case, subsequent development involves a succession of inductive, reciprocal epithelial-mesenchymal interactions (Thesleff et al., 1995). Despite the expression of both PTHrP and the type I receptor during the early stages of development in hair follicles and sebaceous glands, the mammary bud and the tooth germ, however, the initial morphogenesis of these structures clearly does not require the peptide's presence. Rather, it is the subsequent growth and further differentiation of these structures that appears to be critically dependent on PTHrP. At the point of developmental arrest or dysregulation in the rescued knockout mouse, all three sites display a common organization, consisting of a fully differentiated epithelial structure which would normally express PTHrP and, separated by a basement membrane, a surrounding mantle of mesenchymal cells which bear the type I receptor. Given the similarities of the developmental processes in these organs and their common ectodermal origins, it is perhaps not surprising to find the same regulatory molecules involved in their morphogenesis. Mice with a homozygous disruption of the gene for the transcription factors LEF-1 display early defects in the development of teeth, vibrissae/hair and mammary glands (van Genderen et al., 1994). The homeobox containing genes Msx-1 and Msx-2 are expressed in the rudiments of the tooth, mammary gland and hair follicle, and Msx-1 has been shown to be essential for tooth development beyond the bud stage (Satokata and Maas, 1994; Noveen et al., 1995; Friedman and Daniel, 1996). Similarly, the paracrine signaling molecules scatter factor/hepatocyte growth factor and bone morphogenetic protein-4 appear to reprise their respective roles in the morphogenesis of each of these three structures, as well as a number of other organs (Birchmeier and Birchmeier, 1993; Thesleff et al., 1995; Kratochwil et al., 1996, Hogan, 1996, Phippard et al., 1996) In humans, variable defects in development of skin, hair, nails and teeth are manifested in a group of more than 150 clinically distinct heredity disorders known collectively as ectodermal dysplasias. One of the most common types, X-linked anhidrotic ectodermal dysplasia or EDA, is characterized by sparse hair, missing teeth, and a reduction in the number, size and

maturation of sebaceous and sweat glands (Kere et al., 1996). The gene mutated in this disease encodes a small, novel membrane protein which is expressed in the epidermis, the basal layer of the sebaceous gland, and in the outer root sheath cells of hair follicles. PTHrP may thus represent one of a limited number of regulatory molecules which orchestrate these variations on a common morphogenetic theme.

The findings in the skin of the rescued animals are consistent with accelerated differentiation in the epidermis and epidermal appendages in the absence of PTHrP, and thus represent the converse of the retarded differentiation caused by PTHrP overexpression in transgenic skin. This inverse correlation between the degree of PTHrP gene expression and the rate of differentiation is also seen in endochondral bone, where accelerated chondrocyte differentiation and premature ossification prevail in the knockout, while overexpression delays both processes. In this context, PTHrP seems to act as a governor to limit the maximal rate of differentiation, most likely by inducing secondary changes in the expression pattern of mesenchymally-derived growth/differentiation factors or components of the extracellular matrix. In addition, however, the rescued-knockout model revealed an absolute requirement for PTHrP in the processes of tooth eruption and ductal entry and/or growth in the mammary fat pad. Once again the peptide is presumably acting to elicit a requisite biological response from the mesenchyme, but in this case, it functions more as a switch which serves to allow, or disallow, further growth or differentiation. Since transgenic overexpression of PTHrP via the K14 promoter produced no evidence of any abnormalities in tooth eruption or ductal penetration, it appears that it is the simple threshold presence or absence of PTHrP and not its quantitative level of expression that is decisive in this setting.

A number of parallels can be drawn between the seemingly disparate processes of tooth eruption and the entry of the primary duct into the mammary fat pad. Both involve the penetration of an epithelial structure through an encasement of mesenchyme, a process which requires the degradation of the stromal extracellular matrix and basement membrane (Thesleff et al., 1995). Accordingly, matrix metalloproteinases such as stromelysin-1 and gelatinase B

exhibit regulated expression during the morphogenesis of both mammary gland and tooth and, in both cases, remodeling of the stromal matrix involves alterations in the expression patterns of the surface proteoglycan, syndecan-1, and the matrix glycoprotein, tenascin (Birchmeier and Birchmeier, 1993; Witty et al., 1996, Thesleff et al., 1990). Tooth eruption is rendered unique, however, by the need to create a pathway through the overlying bone and its consequent dependence on the activity of osteoclasts.

An intriguing aspect of the defect in osteoclast activation in the rescued PTHrP-knockout animals is its restriction to the microenvironment of the tooth. There is no evidence of osteopetrosis in the skeletons of PTHrP-knockout mice (Karaplis et al., 1994) or in adult PTHrP-null heterozygotes, which display evidence of haplotype insufficiency in trabecular bone with increasing age (Amizuka et al., 1996). Thus, the defect in bone resorption cannot be intrinsic to the osteoclast, but rather must be due to a deficiency in a local signaling molecule. Since osteoclasts do not possess receptors for PTH (or PTHrP), it has been proposed that the promotion of bone resorption by these hormones is mediated indirectly through the release of some paracrine factor from a nearby receptor-bearing cell, such as the osteoblast (Rodan and Martin, 1981). It seems likely that the same type of paracrine cascade applies to the regulation of osteoclasts mediating tooth eruption through alveolar bone. Since the creation of the eruption pathway is a polarized process which is dependent upon the presence of the coronal segment of the dental follicle, type I receptor-bearing cells in the follicle must also be considered as a candidate source for the putative osteoclast-activating factor. Such a cascade would correspond to a true "PTH-like" function for PTHrP in vivo that would be biologically appealing as a functional correlate of the conserved amino terminal domains of the two proteins and their shared use of a single receptor.

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Figure Legends

Figure 1. Gross appearance of rescued PTHrP-knockout mice. (A) Relative size of a rescued-knockout mouse at eight weeks of age as compared to a normal littermate. (B) The female rescued-knockout mouse (right) displays a matted and oily coat, abnormally long nails, and an absence of nipples as indicated by the lack of periareolar markings seen in the littermate (left). (C and D) The chondrodystrophic shape of the cranium in the rescued knockout mouse (D) is readily apparent when contrasted with a normal animal (C). The noticeable protrusion of the eyeballs is likely due to shallow orbits.

Figure 2. Skeletal staining. Neonatal mice were stained with alizarin red S to distinguish calcified tissue. Shown from left to right are examples of wild-type (A), col II-PTHrP transgenic (B), PTHrP-knockout (C) and rescued PTHrP-knockout (D) phenotypes. The PTHrP-knockout animals display inappropriate ossification of the costal cartilage and along the length of the sternum. These abnormalities appear to be largely corrected in the rescued mice (D), in which ossification is confined to the sternebrae and does not extend to the costal cartilage. Only a mild delay in ossification (typically in the pelvis and distal tail) is apparent in the col II-PTHrP transgenic line used in these studies (B). In the magnified views of the cranium (E-H), note the persistence of features typical of cranial chondrodystrophy (domed calvarium, foreshortened snout) in the rescued-knockout animal (H), despite the apparent correction of the accelerated ossification of the endochondral bones of the cranial base which occurs in the knockout (G). The tips of the emerging mandibular incisors can be discerned in both the normal (E) and transgenic (F) animals.

Figure 3. Skin histology. Fixed, full thickness samples of adult dorsal skin were paraffin-embedded, sectioned and stained with hematoxylin-eosin. In contrast to normal skin (A), sections from a

rescued PTHrP-null mouse (B) display: 1) a thinner, hyperkeratotic epidermis with small, flattened keratinocytes; 2) highly keratinized hair follicles, 3) smaller, evacuated sebaceous glands, 4) a fibrotic, cellular dermis, and 5) a reduction in subcutaneous fat. The scale bar represents 20 μm.

Figure 4. Mammary gland development. Whole mounts of the number 4, inguinal mammary glands from four month-old female mice were prepared by resection in toto, fixation in acid-ethanol and staining in carmine red. The normal gland (A) is characterized by a fully branched epithelial duct system surrounding the central lymph node. In the rescued PTHrP-knockout gland (B), however, epithelial structures are completely absent; only the lymph node and the vasculature are present in the fat pad. The scale bar represents 5 mm.

Figure 5. Comparison of adult skulls. Heads from wild-type mice (A and B) and rescued-knockouts (C and D) were skinned and the brains removed prior to digestion by Dermestid beetle larvae for several days. A consequence of PTHrP ablation in the rescued mouse is the failure to develop functional dentition; eternal incisors and molars are clearly absent from both mandible and maxilla in these animals (B and D). This phenotype is superimposed on the underlying features of the cranial chondrodystrophy.

Figure 6. Radiographic analysis of craniofacial growth and tooth eruption. Shown are wild-type littermates (A-C) and rescued PTHrP-knockout mice (D-F) at 1 week (A, D), 2 weeks (B, E), and 3 months of age (F, G), respectively. While the normal mouse displays appropriate craniofacial development, the domed calvarium and foreshortened snout found in the rescued-knockout animal do not ameliorate over time. Also, the normal pattern of tooth eruption, seen in the top row, does not occur in the rescued-knockout mouse. Radiographic densities apparent in the mandible and maxilla of these animals represent progressively impacted teeth.

Figure 7. Tooth histology. (A and B) Sagittal, non-decalcified methacrylate-embedded sections of neonatal incisors from a rescued PTHrP-knockout mouse (B) and a wild-type littermate (A) stained with toluidine blue. A well developed ameloblast layer (fringe-like, columnar cells) is readily apparent on the labial aspect (upper, as positioned here) of the normal tooth, but is lacking in the mutant, which appears choked by the surrounding alveolar bone. The darkly-stained layer outlining the incisor is dentin and the adjacent lightly-stained material is enamel (best observed on the labial surface of the normal incisor); the space between the ameloblast layer and the enamel is a sectioning artifact. (C and D) mandibles from one-week-old wild-type (C) and rescued-knockout mice (D) were decalcified in EDTA and then sectioned sagittally through the molar crypt. Distortion of the teeth in the rescued-knockout animal due to progressive impaction is readily apparent. (E) Magnification of a sagittal section through the region between the incisor and the first molar of a mandible from a rescued-knockout animal. Osteoclasts (arrowheads) can be seen attached to the surfaces of the alveolar bone and do not appear to be reduced in number. (F) Coronal sections of mandibles from neonatal rescued-knockout animals (controls included wild-type, col II-PTHrP transgenics, PTHrP-knockouts and op/op animals, not shown) were stained for TRAP activity. TRAP staining is apparent in the alveolar bone immediately adjacent to the lateral aspect of the molars. The scale bars represent 64 μm in A and B, 32 μm in C, D and F, and 16 μm in E.

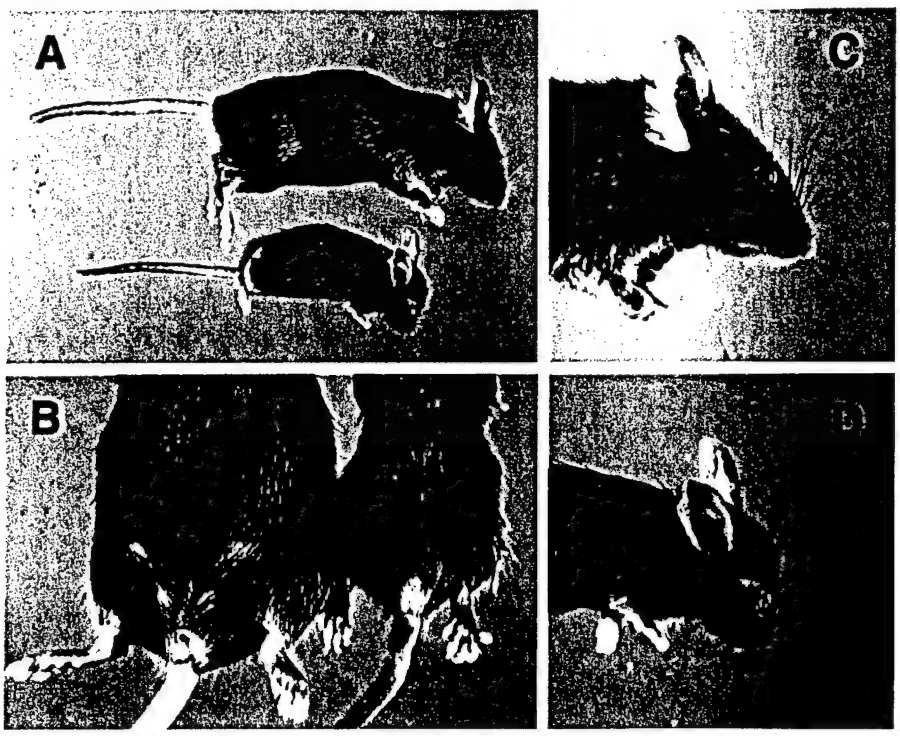
Figure 8. Coat appearance and skin histology in doubly-rescued PTHrP-knockout mice. Col II-PTHrP rescued-knockout mice (A) and K14-PTHrP/col II-PTHrP doubly-rescued knockout mice (B) are similar in overall size and shape, but the doubly-rescued animal shows a normalization in the appearance of the coat and substantial correction (C) of the pathological features found in the PTHrP-deficient skin (compare to Fig. 2). The scale bar represents 20 μm.

Figure 9. Whole mount histology of mammary glands in doubly-rescued PTHrP-knockout mice. Incipient ductal growth can be detected in the extreme right end of the number 4 gland from an adolescent doubly-rescued female (A) and is better seen in

magnification (B). Following the induction of sexual maturation with estrogen and progesterone, full ductal growth is obtained (C) relative to wild-type (D). The scale bar represents 5 mm in A and B, and 1 mm in C.

Figure 10. Dental phenotype of doubly-rescued PTHrP-knockout mice. (A) Immunohistochemical staining for K14 in a sagittal section from a normal neonatal incisor localizes to the reduced enamel epithelium on the labial surface. Similar staining surrounds the molar crowns (not shown). (B) Misalignment and overgrowth of the mandibular incisors is apparent in an adult doubly-rescued animal. (C) Sagittal radiographs of adult mice reveal the malocclusion due to the underlying chondrodystrophy in the rescued-knockout (bottom). Also shown are normal (top) and PTHrP-knockout animals (middle). The scale bar represents 27 μm.

Figure 1



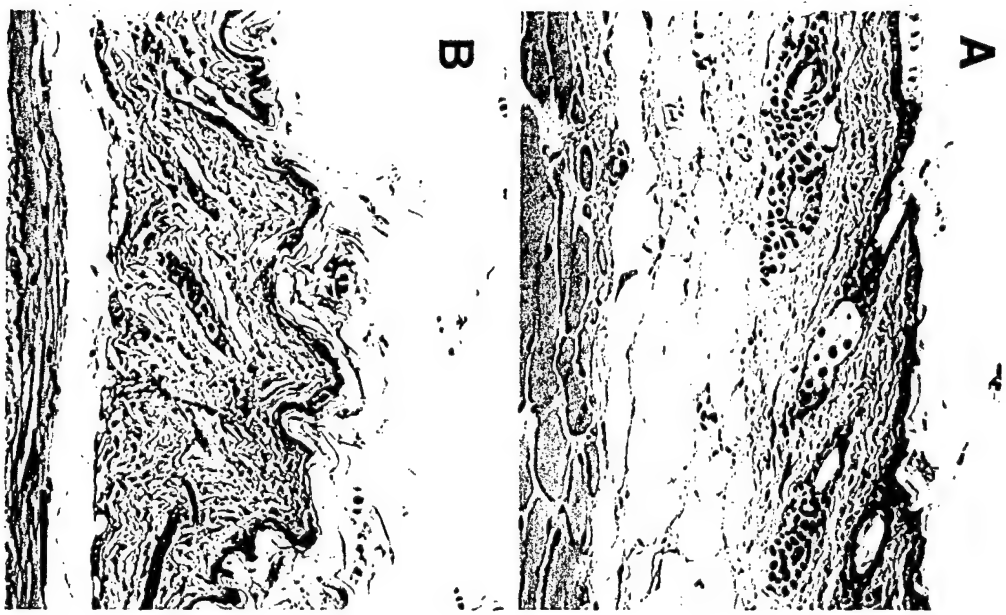
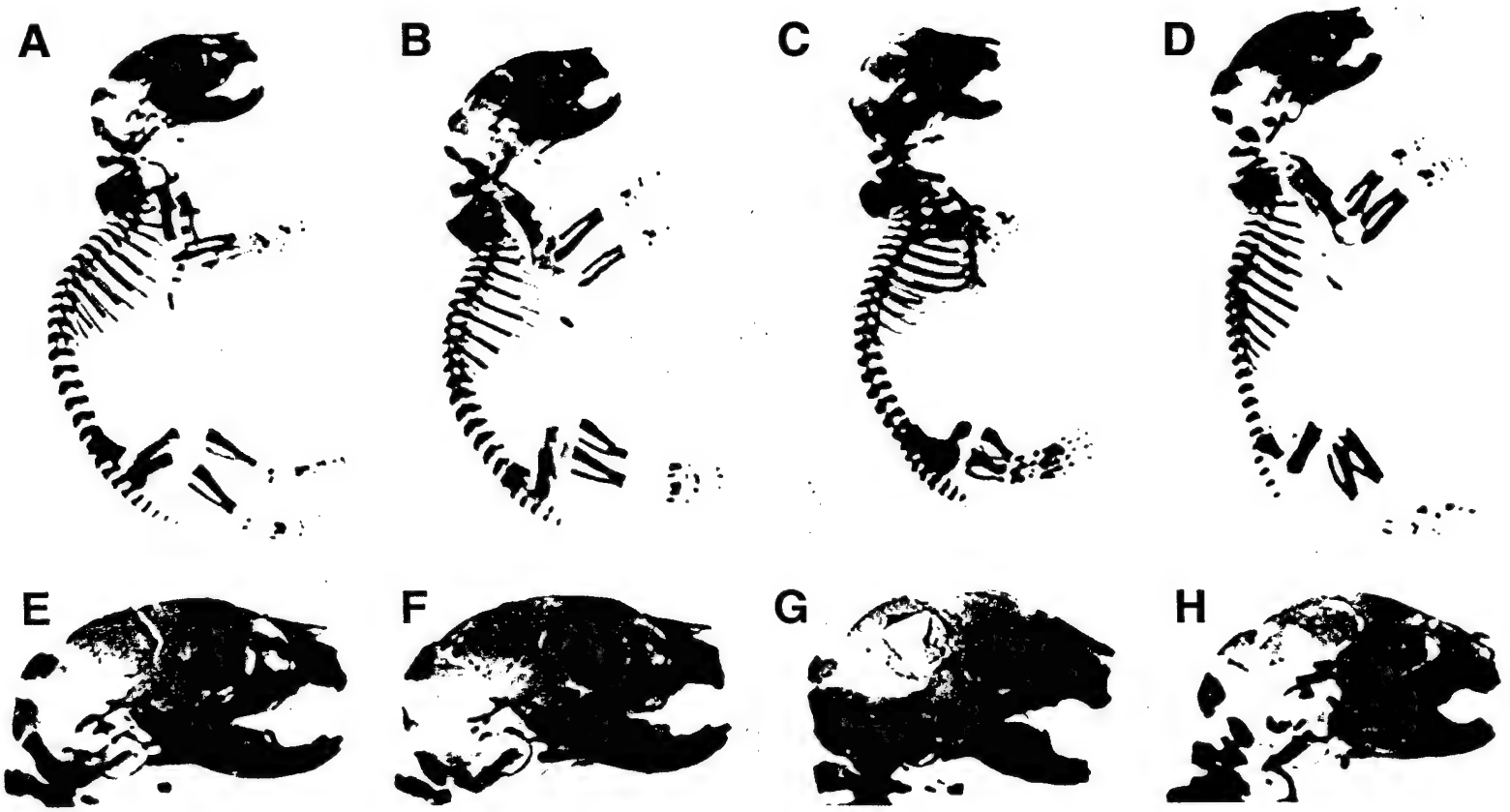


Figure 3

Figure 2



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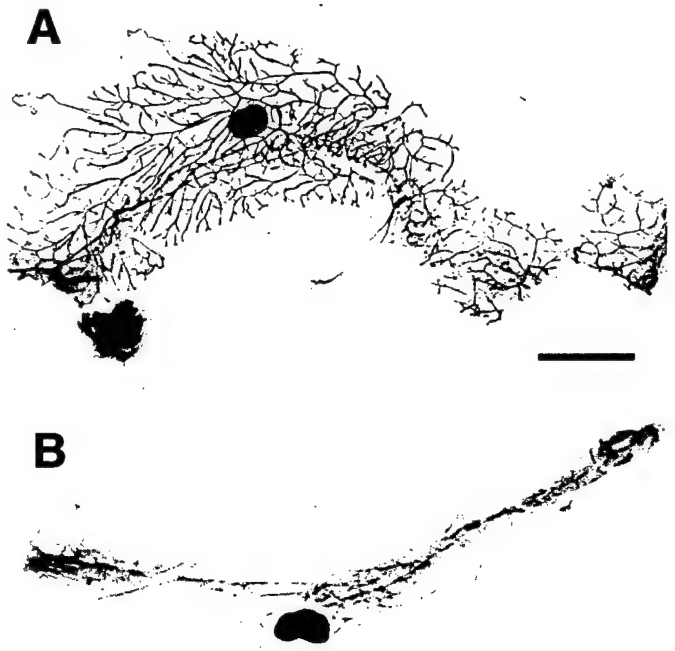


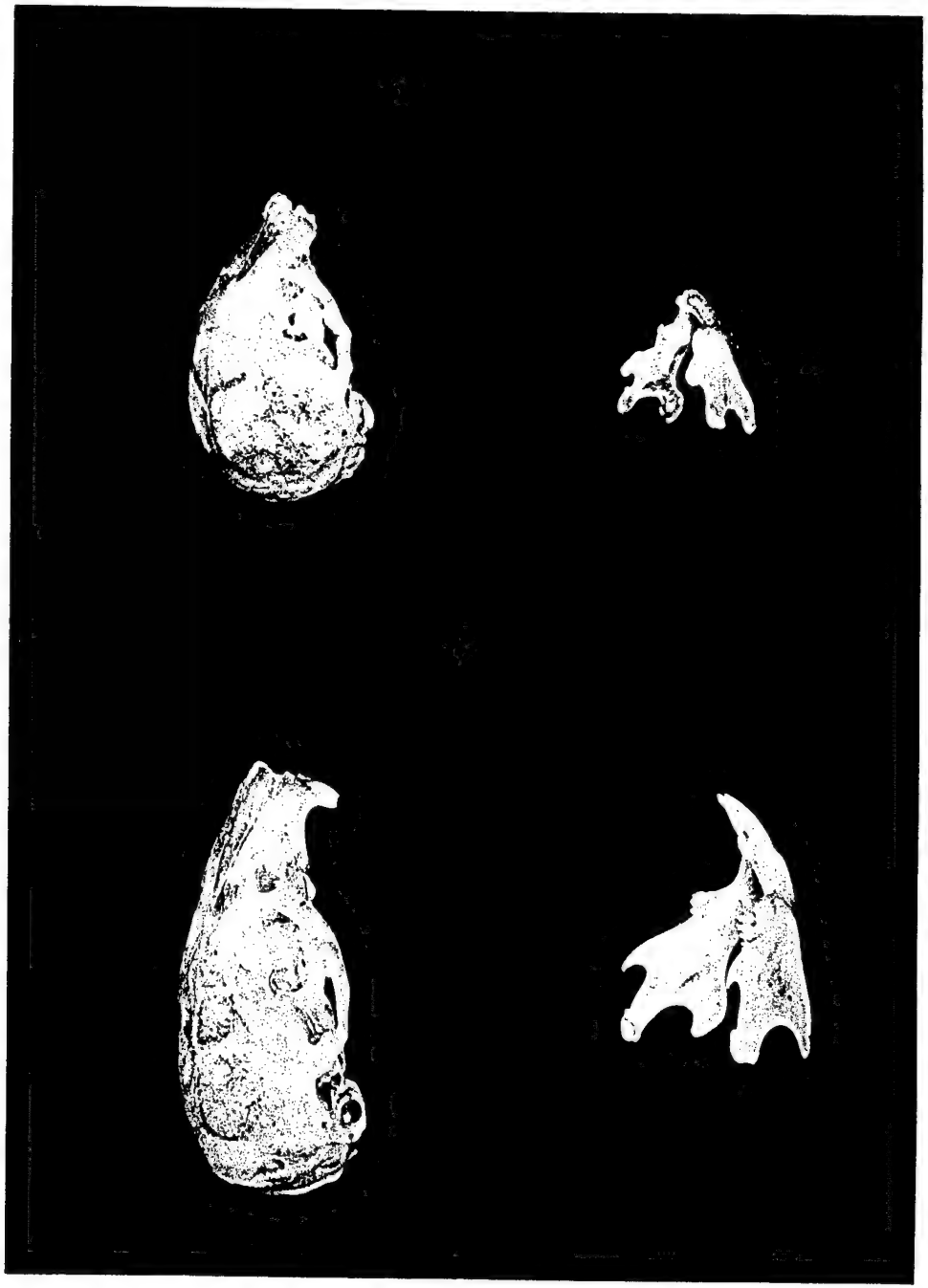
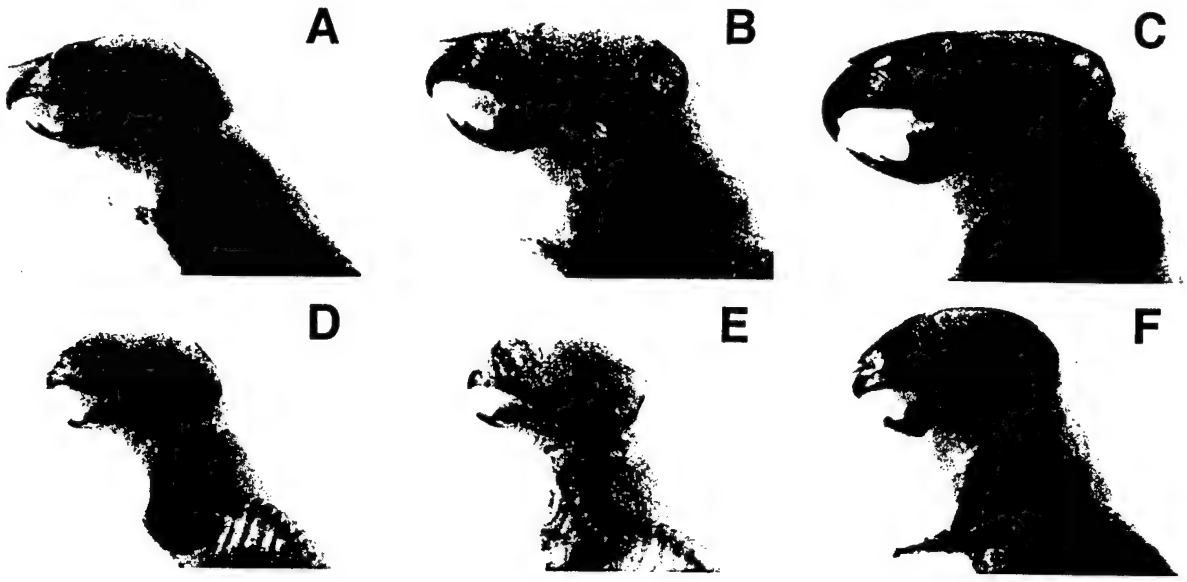


Figure 5

Figure 6



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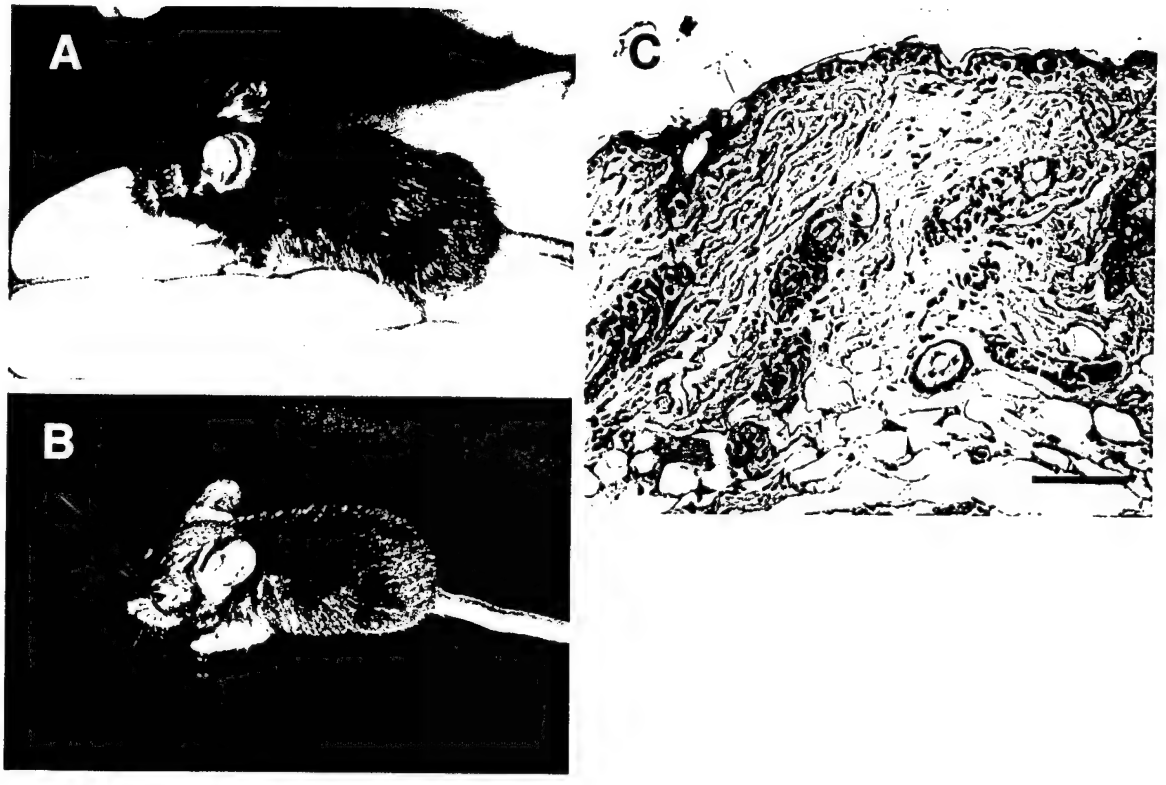


Figure 7

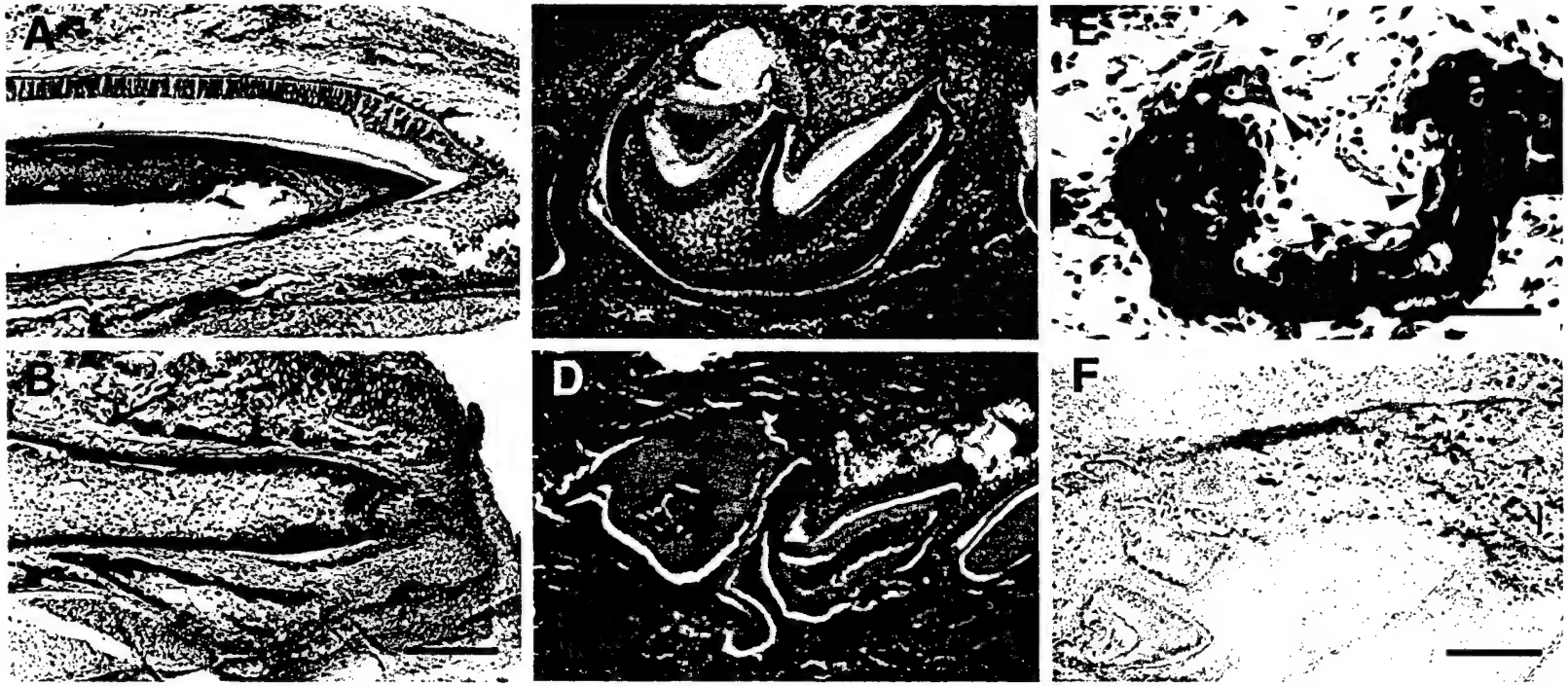


Figure 9

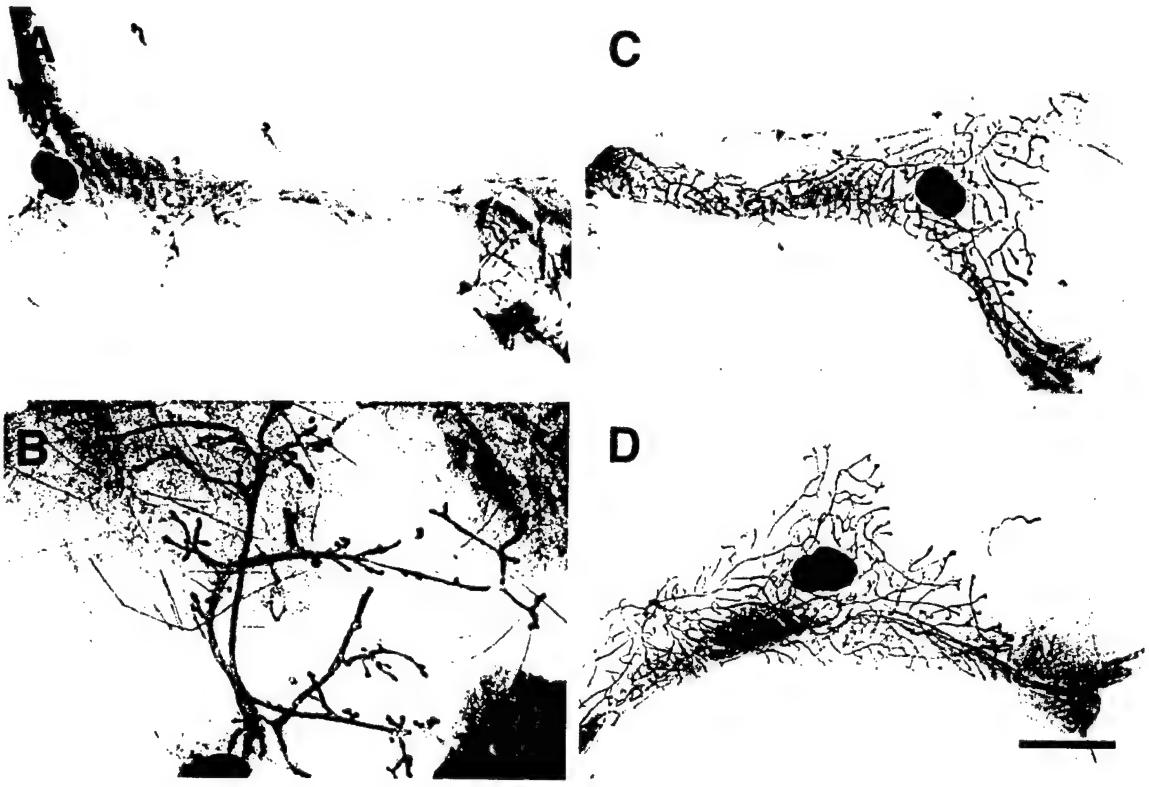
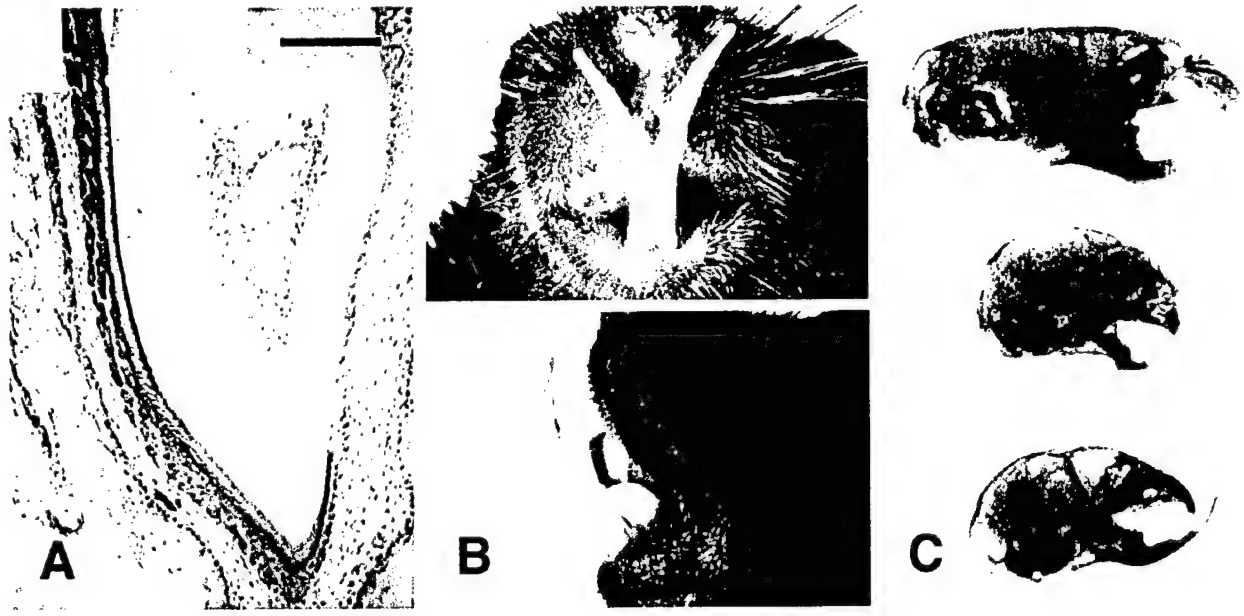


Figure 10



PARATHYROID HORMONE-RELATED PROTEIN IS REQUIRED FOR EMBRYONIC MAMMARY GLAND DEVELOPMENT

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Key Words: epithelial-mesenchymal interactions, branching morphogenesis, gene targeting, transgenic mice, keratin 14, organogenesis, PTH/PTHrP receptor, mammary mesenchyme

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Summary

Parathyroid hormone-related protein (PTHrP) was originally discovered as a tumor product that causes humoral hypercalcemia of malignancy. PTHrP is now known to be widely expressed in normal tissues, and growing evidence suggests that it is an important developmental regulatory molecule. We had previously reported that overexpression of PTHrP in the mammary glands of transgenic mice impaired branching morphogenesis during sexual maturity and early pregnancy. We now demonstrate that PTHrP plays a critical role in the epithelial-mesenchymal communications that guide the initial round of branching morphogenesis that occurs during the embryonic development of the mammary gland. Disruption of the PTHrP gene leads to a failure of the initial round of branching growth responsible for transforming the mammary bud into the rudimentary mammary duct system. In the absence of PTHrP, the mammary epithelial cells degenerate and disappear. The ability of PTHrP to support embryonic mammary development is a function of amino-terminal PTHrP, acting via the PTH/PTHrP receptor, for ablation of the PTH/PTHrP receptor gene recapitulates the phenotype of PTHrP gene ablation. We have localized PTHrP expression to the embryonic mammary epithelial cells and PTH/PTHrP receptor expression to the mammary mesenchyme using in situ hybridization histochemistry. Finally, we have rescued mammary gland development in PTHrP-null animals by transgenic expression of PTHrP in embryonic mammary epithelial cells. We conclude that PTHrP is a critical epithelial signal that is received by the mammary mesenchyme and that is involved in supporting the initial round of branching morphogenesis that transforms the mammary bud into the primary epithelial duct system.

Introduction

Parathyroid hormone-related peptide (PTHrP) was initially isolated from tumors causing the paraneoplastic syndrome of humoral hypercalcemia of malignancy (HHM) (Wysolmerski and Broadus, 1994). Its name reflects the fact that PTHrP and parathyroid hormone (PTH) are the products of genes that have diverged from a common ancestor through a process of gene duplication (Broadus and Stewart, 1994). Unlike PTH, which is produced only by the parathyroid glands and circulates as a classic peptide hormone that regulates systemic calcium metabolism, PTHrP is produced by a wide variety of fetal and adult tissues, does not circulate, and exerts its actions locally (Broadus and Stewart, 1994). PTH and PTHrP retain a high degree of homology in their amino-terminal portions, and PTH and amino-terminal species of PTHrP have retained the use of a common G-protein-coupled receptor, the PTH/PTHrP receptor (Jüppner et al., 1991). PTHrP has also been shown to undergo post-translational processing to generate several other peptides, at least one of which has been demonstrated to have biological activity subserved by an as yet unidentified receptor distinct from the PTH/PTHrP receptor (Wu et. al., 1996; Kovacs et al., 1996).

PTHrP has been implicated in the regulation of a variety of biological processes such as cell growth and differentiation, the regulation of pancreatic islet cell function, the regulation of smooth muscle tone and the facilitation of placental calcium transport (Philbrick et al., 1996). Although the exact physiological functions of PTHrP remain unclear in mature organisms, a series of experiments in transgenic mice over the past several years has demonstrated that PTHrP serves important roles during fetal development. Studies in mice

homozygous for PTHrP and PTH/PTHrP receptor gene disruptions as well as in mice overexpressing PTHrP in chondrocytes have shown that PTHrP regulates the program of chondrocyte differentiation during endochondral bone formation (Karaplis et al., 1994; Vortkamp et al., 1996; Lanske et al., 1996; Weir et al. 1996). In addition, overexpression of PTHrP in keratinocytes (Wysolmerski et al., 1994) and mammary myoepithelial cells (Wysolmerski et al., 1995) has been shown to impair hair follicle and mammary gland formation, respectively. Finally, as described in the companion manuscript (Philbrick et al., 1997), in addition to the skeletal abnormalities reported previously, disruption of the PTHrP gene also leads to multiple defects in ectodermal development. PTHrP-knockout mice usually die from their skeletal abnormalities just after birth, but Philbrick et. al. (1997) have succeeded in rescuing PTHrP-knockout mice from this neonatal death by placing a procollagen II - PTHrP transgene onto the homozygous PTHrP-null background (Weir et al. 1996; Philbrick et al., 1997). The resulting mice (col II-PTHrP/PTHrP-null mice) survive into adult life, but have defects in tooth, skin, sebaceous gland and mammary gland development.

Shortly after its discovery, PTHrP mRNA was found to be expressed in the lactating mammary gland, and PTHrP was found in high concentrations in milk (Thiede and Rodan, 1988; Budayr et al., 1989). It is now clear that PTHrP is expressed at various stages during mammary gland development, although whole gland mRNA expression appears to peak during pregnancy and lactation, when PTHrP gene expression is stimulated by circulating prolactin (Thiede and Rodan, 1988; Philbrick et al., 1996). The role of PTHrP during lactation remains obscure, but during sexual maturation and in early pregnancy PTHrP has been suggested to play a role in modulating branching morphogenesis (Wysolmerski et al., 1995). Overexpression of PTHrP in myoepithelial cells has been shown to retard ductular growth and to impair side

branching during sexual maturation as well as to inhibit the formation of terminal ductules during early pregnancy (Wysolmerski et al., 1995). In addition, PTHrP introduced directly into the mammary fat pads of normal mice has been shown to impair estrogen- and progesterone-induced ductular proliferation (Wysolmerski et al., 1995). The critical role of PTHrP in the development of the mammary epithelial duct system is underscored by the mammary phenotype of the aforementioned col II-PTHrP/PTHrP-null mice. These mice do not express PTHrP in their mammary glands, and they are completely devoid of mammary epithelial ducts or nipples (Philbrick et al., 1997). These findings suggest that there is an absolute requirement for PTHrP in the formation of the mammary epithelium. Because the initial mammary ductal system and nipples are formed before birth (Sakakura, 1987), we examined mammary development in PTHrP-knockout embryos in order to characterize the mechanisms by which the lack of PTHrP leads to the failure of mammary ductal development. In this report we demonstrate that PTHrP is a critical epithelial signal that is required in order for the mammary mesenchyme to facilitate the initial round of branching morphogenesis from the mammary bud.

Materials and Methods

Mouse strains and identification of knockout embryos.

The disrupted PTHrP allele (Karaplis et al., 1994) was progressively outbred onto a CD-1 background, and mice heterozygous for this allele were mated to produce PTHrP-null embryos of various ages. The date of the appearance of a vaginal plug was considered to be day 0 of embryonic life. Embryos were removed from the uterus and genotyped with respect to the presence or absence of neo gene sequences and the presence or absence of

an intact PTHrP coding region (exon IV) by PCR, utilizing primers as previously described (Philbrick et al. 1997). This allowed the identification of wild-type, heterożygous and homozygous PTHrP-null embryos.

The disrupted PTH/PTHrP receptor allele was progressively bred onto a Black Swiss background, and homozygous-null embryos were produced and identified in like fashion (Lanske et al., 1996). The PTH/PTHrP receptor primer pair utilized for this purpose amplified a 270-bp portion of the PTH/PTHrP gene and consisted of the following sequences: forward 5'GCAGAGATTAGGAAGTCTTGGA and reverse 5'AGCCGTCGTCCTTGGGAACTGT.

K14-PTHrP/PTHrP-null embryos were produced by mating K14-PTHrP transgenic hemizygotes (Wysolmerski et al. 1994) with mice heterozygous for the PTHrP-null mutation. Offspring of this cross that were both hemizygous for the K14-PTHrP transgene and heterozygous for the PTHrP-null gene were then crossed to mice heterozygous for the PTHrP-null allele to produce mice homozygous for a disrupted PTHrP gene and hemizygous for the K14-PTHrP transgene. The K14 transgene was identified as previously described (Wysolmerski et. al., 1995). Each of the various types of embryos was also sexed based on the presence or absence of a 240-bp band amplified from the SRY gene using the following primers: forward 5' CGGGATCCATGTCAAGCGCCCCATGAATGCATTTATG and reverse 5'GCGGAATTCACTTTAGCCCTCCGATGAGGCTGATAT (Giese et al., 1994).

Histology/Immunohistochemistry

Embryos were harvested by cesarean section and fixed in 4% paraformaldehyde at 4°C for 12 hrs. The ventral skin was then removed, and the embryonic mammary glands were identified using transmitted light and photographed under low magnification. Subsequently, the mammary glands

were dissected from the ventral skin and embedded in paraffin. Serial 5 micron sections were cut and stained with hematoxlyn and eosin for microscopic examination. Immunohistochemistry was performed using standard techniques. The K-14 antibody is an affinity-purified rabbit polyclonal antibody and was used at dilutions of 1:500 or 1:1000 (Wysolmerski et al., 1995). The mouse casein antibody is a rabbit polyclonal antibody (kind gift of B Vonderhaar, NIH, Bethesda MD) and was used at a dilution of 1:200. All primary incubations were performed for 12 hrs. at 4° C, and primary antibody binding was detected using the Vector Elite avidin-biotin kit (Vector Laboratories, Burlingame, CA) and 3, 3' diaminobenzidine as a chromagen. Slides were counterstained using hematoxlyn. Apoptosis was detected by terminal deoxytransferase labeling (TUNEL assay) employing the In Situ Cell Death Detection Kit from Boehringer Mannheim (Mannheim, Germany).

In situ hybridization histochemistry

In situ hybridization histochemistry was performed on 5 micron paraffin sections of embryonic mammary glands using a modification of a previously described protocol (Zhang et al., 1995). Probes corresponded to a 349-bp genomic fragment of the mouse PTHrP gene and a 238-bp cDNA fragment of the PTH/PTHrP receptor gene, as previously described (Weir et al., 1996). Sense and antisense riboprobes were generated from linearized fragments using an in vitro transcription kit (Promega, Madison, WI) in the presence of ^{35}S-UTP (1000Ci/mmol, Amersham, Life Science, Arlington Heights, IL). Before hybridization, sections were dewaxed and rehydrated, treated with proteinase K (3 ug/ml in PBS for 17 min at room temp), and acetylated with 0.25% acetic anhydride in the presence of 0.1M triethanolamine/0.9% NaCl (pH 8.0) for 10 min. Sections were then rinsed in 2x SSC and incubated for 30 min in 0.66% N-ethylmaleimide (Sigma Chemical Co., St Louis, Mo) in 2x SSC,

rinsed again in 2xSSC, dehydrated in graded alcohol, treated with chloroform for 5 min, rehydrated and then air dried. The probes ($1.5x10^7$ cpm/ml) were then hybridized to the samples for 17 hrs at 54°C in a humidified chamber. Hybridization buffer consisted of 50% formamide, 10% dextran sulfate, 1x Denhardt's solution, 4x SSC, 250 ug/ml tRNA, 100 ug/ml salmon sperm DNA, and 50 mM DTT. After hybridization, sections were rinsed in 1x SSC and washed twice in 2x SSC/50% formamide for 5 min at 52°C, rinsed in 2x SSC, and treated with 30 ug/ml RNase A in 2x SSC at 37°C for 30 min. Following two rinses in 2x SSC, sections were again washed in 2x SSC/50% formamide at 52°C for 5 min, dehydrated through graded ethanol, air dried and dipped in a 1:1 mixture of NTB-2 (Kodak) photographic emulsion and water and exposed at 4°C for 3 weeks. After development, sections were counterstained with hematoxylin and mounted for microscopic examination.

Results

Loss of PTHrP results in a failure of the mammary epithelial primary growth spurt.

In mice, the mammary glands first develop on E 10-11 as ridge-like thickenings of the ventral epidermis known as the mammary streaks, which extend from the anterior limb buds to the posterior limb buds bilaterally. From E12 - 14, epidermal cells within these ridges migrate to specific locations to form the five pairs of mammary anlage or buds. In female mice, the mammary buds remain relatively quiescent until E 16 when they begin an initial round of branching morphogenesis, elongating and penetrating into the developing mammary fat pad. This process is referred to as the primary growth spurt and, by birth, it results in the formation of a mammary duct structure with approximately 15-20 branches, a pattern that remains until a second round of

branching growth occurs during sexual maturation (Sakakura, 1987). The nipples of mice are formed at E 18 as a circular invagination of the epidermis, referred to as the nipple sheath, which surrounds the primary duct of the developing gland (Sakakura et al., 1987). Philbrick and colleagues have shown that col II-PTHrP/PTHrP-null mice, which express PTHrP only in chondrocytes, lack mammary epithelial ducts and nipples (Philbrick et al., 1997). Given the timing of nipple formation within the developmental sequence outlined above, we reasoned that the loss of epithelial ducts resulting from the lack of PTHrP most likely occurred during the embryonic phase of mammary gland development. Therefore, we examined the various phases of embryonic mammary gland growth in PTHrP-knockout embryos at days E 12-13, E 15, E 18, and at birth.

Figs 1 and 2 demonstrate the gross and microscopic appearance, respectively, of the mammary rudiments from mice homozygous for a disrupted PTHrP gene as compared to their wild-type littermates. As can be seen in Figs 1 A&B and Figs 2 A&B, the mammary buds appeared to have formed normally in PTHrP-null embryos at E 15. This was also the case at E12-13 (data not shown). In contrast, there was a dramatic difference in the appearance of the PTHrP-knockout ducts as compared to those in wild-type embryos at E 18. As seen in Figs. 1C & D, in the normal embryos the mammary bud has given rise to a primary duct which has elongated to make contact with the developing mammary fat pad and has formed several initial branches. In the knockout embryos, the mammary buds failed to make this transition and appeared similar to those at E 15 (Figs. 1 E & F) Furthermore, the mammary fat pads, although present, appeared somewhat diminished in size in the knockout embryos.

On microscopic examination, one could see that by E18 the normal ducts had undergone a growth spurt, had extended into the lower dermis and

had formed initial branches that could be seen amongst the preadipocytes constituting the developing mammary fat pad (see Figs 2C&D). At this point, the normal mammary glands also had well developed nipple sheaths surrounding the origins of the primary ducts (Fig. 2C). In contrast, as seen in Fig. 2E, at E18 the PTHrP-knockout ducts appeared not to have undergone the primary growth spurt. Instead of extending to the fat pad and branching, epithelial ducts were uniformly found only in the upper portions of the dermis, where they were enveloped by a dense condensation of fibroconnective tissue. In addition, there was no evidence of nipple sheath development surrounding the origins of the epithelial ducts in the PTHrP-knockout embryos. When examined at higher magnification, the epithelial cells within the knockout ducts often appeared to be degenerating. As compared to normal epithelial cells (Fig. 2F), there was separation of the PTHrP-knockout epithelial cells (Fig. 2G) from the basement membrane, the cells borders were indistinct, and many nuclei appeared pycnotic. Consistent with this observation, by birth, there were only scattered remnants of degenerating mammary ducts that could be found on serial sectioning of the PTHrP-null embryos, while in wild type embryos the mammary ducts were firmly established within the mammary fat pad and had developed the expected branching pattern (data not shown). In summary, in the absence of PTHrP, mammary development proceeds normally through the mammary bud stage but subsequently falters as the buds fail to undergo the initial phase of branching morphogenesis, and the mammary epithelial cells subsequently degenerate.

PTHrP has been shown to delay chondrocyte differentiation and apoptosis, and disruption of the PTHrP gene results in a form of growth failure associated with premature differentiation and apoptosis of chondrocytes in the growth plate of developing bones (Weir et al., 1996; Amling et al., 1997;

Karaplis et al., 1994; Lee et al., 1996; Vortkamp et al., 1996). Given this information and the apparent degeneration of the mammary epithelial cells in the PTHrP-knockout embryos, we next examined these cells for evidence of apoptosis and/or inappropriate differentiation at E18 by TUNEL assay and by immunohistochemistry for ß-casein. Despite the histological appearance of the knockout epithelial cells, there were no apoptotic cells within these ducts as determined by TUNEL staining (Fig. 3B). In contrast, TUNEL staining of normal littermate mammary rudiments (Fig. 3A) revealed frequent apoptotic cells located within the center of the mammary ducts, presumably associated with lumen formation (Coucouvanis and Martin, 1995). Likewise, immunohistochemistry for ß-casein revealed no evidence that the knockout mammary epithelial cells were undergoing premature cytodifferentiation; there was no staining for ß-casein in either normal or knockout epithelial cells at E18 (data not shown). These data suggest that, unlike the events in chondrocytes, the failure of mammary development in PTHrP-knockout mice appeared neither to be associated with premature differentiation of the mammary epithelial cells nor with widespread apoptosis of these cells.

Ablation of the PTH/PTHrP receptor gene recapitulates the mammary phenotype of PTHrP-knockout mice.

As mentioned in the Introduction, through a series of post-translational processing steps, the PTHrP gene gives rise to a family of proteins, several of which have now been shown to have specific biological functions (Broadus and Stewart, 1994; Wu et al., 1996). In addition, it has been suggested that PTHrP may be targeted to the nucleus and exert biological activity via an intracrine pathway (Henderson et al., 1995). Since previous experiments had implicated soluble, amino-terminal PTHrP acting via the PTH/PTHrP receptor as important

in the regulation of branching morphogenesis in the mammary gland during sexual maturation and pregnancy (Wysolmerski et al. 1995), we hypothesized that the same ligand/receptor pair might well be operative during fetal mammary gland development. In order to test this hypothesis, we examined mammary gland development in PTH/PTHrP receptor-null embryos (Lanske et al., 1996) over the same time frame as in the PTHrP-null embryos in order to see if ablation of this receptor would have the same consequences as ablation of PTHrP itself.

Fig. 4 demonstrates the appearance of the mammary rudiment in PTH/PTHrP receptor-knockout mice and control littermates. As seen in the PTHrP-knockout embryos, in the receptor-knockout mice the primary round of branching morphogenesis failed, leading to the subsequent degeneration of the mammary epithelial ducts. Just as with the PTHrP knockouts, the mammary buds appeared to form appropriately in the receptor-knockout mice (data not shown), but clear differences in the appearance of the receptor-knockout mammary rudiment as compared to normal littermates were apparent by E18. As shown in Fig. 4, by E18, the normal duct system (Fig. 4 A&D) had grown to the fat pad and begun to branch, while the knockout mammary duct failed to elongate or branch and remained bud-like in its appearance (Fig. 4 B). Examination at higher magnifications revealed that the mammary ducts in the receptor-knockout mice (Fig. 4 C&E) remained in the upper dermis, were enveloped within an abnormal condensation of stroma, and appeared to be degenerating, a picture nearly identical to that seen with mammary ducts devoid of PTHrP (see Fig. 2). Furthermore, as in the absence of PTHrP, the receptor-knockout embryos formed no nipple sheath (see Fig. 4 B). Therefore, ablation of PTHrP or the PTH/PTHrP receptor led to the same phenotype, a failure of the

initial phase of branching morphogenesis during embryonic mammary development.

Localization of PTHrP and PTH/PTHrP receptor gene expression during embryonic mammary gland development.

In order to begin to study the mechanisms by which PTHrP participates in the regulation of embryonic mammary development, we determined the sites of PTHrP and PTH/PTHrP gene expression in normal Balb C mammary rudiments from E12 through E18 by in situ hybridization. As shown in Fig. 5 A-C, PTHrP mRNA expression in the developing mammary rudiment was limited to the epithelial cells, especially those cells located peripherally, adjacent to the basement membrane. As reported previously (Lee et al., 1995), PTHrP mRNA was also detected in keratinocytes within the epidermis as well as within developing hair follicles, although it appeared that there were higher levels of expression within the mammary epithelial structures than within either the epidermis or hair follicles. In addition, expression of the PTHrP gene did not appear to be induced at any specific time point during the time period that we examined (E12- 18). Rather, PTHrP mRNA was continuously expressed at high levels in mammary epithelial cells in the mammary bud as well as in the growing ducts during the initial phase of branching morphogenesis.

In contrast to the epithelial expression pattern seen for PTHrP, expression of the PTH/PTHrP receptor was limited to the mesenchyme surrounding the developing mammary ducts. As seen in Fig 5 D-F, PTH/PTHrP receptor mRNA was expressed throughout the embryonic dermis, including the dense mammary mesenchyme. At E12 - 13, the expression of the receptor mRNA appeared to be fairly uniform throughout the dermal mesenchyme (data not shown), but, from E15 onward, there appeared to be more intense

hybridization of the receptor antisense probe in the upper, more cellular dermis (Fig. 5 E). At E18, at a point at which the mammary ducts had grown to make contact with the mammary fat pad, PTH/PTHrP receptor mRNA continued to be expressed in the stromal cells surrounding the growing mammary ducts as they became surrounded by the developing fatty stroma (data not shown). As with PTHrP gene expression, the PTH/PTHrP receptor gene was expressed throughout the time frame examined, and there was not a specific point at which its expression appeared to be induced. Therefore, within the embryonic mammary gland, PTHrP and the PTH/PTHrP receptor appear to represent an epithelial/mesenchymal signaling unit in which PTHrP is produced by mammary epithelial cells and interacts with its receptor on mammary mesenchymal cells. Furthermore, both the PTHrP and the PTH/PTHrP receptor genes appear to be expressed constitutively within the developing fetal mammary gland.

Transgenic expression of PTHrP rescues the mammary glands of PTHrP-knockout mice.

We hypothesized that the failure of mammary development seen in the PTHrP and PTH/PTHrP receptor-knockout embryos was due to the loss of PTHrP-mediated paracrine signaling between the mammary epithelium and mammary mesenchyme at the time of the transition of the mammary bud to its initial round of branching morphogenesis. This working hypothesis suggested that reintroducing PTHrP into the local microenvironment of the mammary bud at this point might prevent the failure of mammary development in these mice. Keratin-14 expression is known to be induced in embryonic skin beginning at E15-16 (Kopan and Fuchs, 1989), about the time of the primary growth spurt of the mammary rudiment. Furthermore, although its pattern of expression in the embryonic mammary gland was not described, the keratin-14 gene has been

shown to be expressed in myoepithelial cells in the adult mammary gland (Smith et al., 1990; Wysolmerski et al., 1995). In the hopes of using transgenic mice expressing the PTHrP gene under the control of the human K14 promoter (K14-PTHrP mice) as a vehicle to reintroduce PTHrP into the mammary environment of the PTHrP-null mice, we examined K14 expression in the embryonic mammary glands of normal mice by immunohistochemistry. As shown in Fig. 6, K14 is expressed uniformly in embryonic mammary epithelial cells beginning around E15. Because this expression pattern appeared to be ideal for our purposes, we bred K14-PTHrP transgenic mice to mice heterozygous for a disrupted PTHrP allele in order to produce mice hemizygous for the K14-PTHrP transgene and heterozygous for a disrupted PTHrP gene. These mice were subsequently crossed to each other to place the K14-PTHrP transgene onto a homozygous PTHrP-null background; the resultant K14-PTHrP/PTHrP-null mice were devoid of PTHrP in all tissues except for those expressing the K14 gene.

As expected, the K14-PTHrP/PTHrP-null mice died at birth due to the skeletal abnormalities resulting from the lack of chondrocyte PTHrP expression but, as opposed to the original PTHrP-knockout mice, these mice had mammary glands. As described in the previous sections, by birth, the epithelial duct system in the PTHrP-knockout embryos had completely degenerated. However, as seen in Fig. 7, at birth, the K14-PTHrP/PTHrP-null mice had a well-formed primary duct that extended into the mammary fat pad and formed the expected initial branches. On H&E section, one could see that the epithelial duct system in the K14-PTHrP/PTHrP-null mice had extended below the upper dermis and had formed normal-appearing secondary ducts within the fatty stroma of the mammary fat pad (see Fig. 7C). Of note, despite the normal appearance of the ductal tree there remained no nipple sheath, as was also the

case in the PTHrP-null embryos (compare Fig. 2E and Fig. 7C). Therefore, expression of PTHrP in the embryonic mammary cells of PTHrP-null embryos under the control of the K14 promoter allowed the mammary bud to undergo the primary growth spurt but did not rescue nipple sheath formation.

Discussion

This report and the companion paper by Philbrick and colleagues record a series of observations that clearly demonstrate that amino-terminal PTHrP is required for the development of the mammary epithelial duct system in mice. First, col II-PTHrP/PTHrP-null mice (devoid of PTHrP in all tissues except for cartilage, see accompanying manuscript) lack all mammary epithelial ducts. Second, in PTHrP-knockout embryos, we found a primary failure of branching morphogenesis during embryonic mammary gland development. Third, deletion of the PTH/PTHrP receptor recapitulated the failure of mammary development seen in the PTHrP-knockout embryos. Finally, reintroduction of PTHrP into mammary epithelial cells by breeding the K14-PTHrP transgene onto a homozygous PTHrP-null background rescued the failure of mammary development seen in the PTHrP-knockout embryos.

The formation of the embryonic mammary gland occurs in two steps: first, the formation of the mammary bud and, second, the initiation of branching morphogenesis leading to the formation of the immature ductal tree (Sakakura, 1987). In PTHrP-knockout embryos, the mammary buds formed appropriately, but they failed to transition successfully into the initial round of branching growth that leads to the typical immature ductal tree. In the absence of PTHrP, the mammary epithelial structures failed to elongate and/or penetrate into the developing fat pad, remaining in the upper dermis and becoming surrounded by a dense condensation of fibroconnective tissue. The mammary epithelial

cells subsequently degenerated; the nipple sheath failed to form, and, by birth, all traces of the mammary epithelial duct system disappeared, explaining the lack of mammary structures in the mature col II-PTHrP/PTHrP-null mice. The exact nature of the epithelial cell degeneration in the PTHrP-knockout embryos remains unclear. PTHrP has been shown to regulate chondrocyte differentiation and apoptosis in the developing growth plate (Weir et al., 1996; Amling et al., 1997; Lee et al., 1996; Vortkamp et al., 1996). However, the loss of the mammary epithelial cells in PTHrP-null embryos did not appear to be associated with either their premature differentiation (as assessed by ß-casein expression) or apoptosis. Histologically, the stromal condensation around the degenerating ducts in the PTHrP-null mice is reminiscent of the androgen-mediated stromal reaction that leads to the deterioration of the mammary rudiment in male embryos (Sakakura, 1987; Kratochwil and Schwartz, 1976). Despite this similarity, in female knockout embryos the mammary buds appeared normal through E15, the point at which the mammary buds in normal male littermates have already largely disappeared. This asynchrony makes it unlikely that modulation of PTHrP secretion and/or PTH/PTHrP receptor signaling is a central feature of the response of the mammary bud to fetal androgens. However, it remains a possibility that alterations in PTHrP signaling might play some role in the deterioration of the mammary epithelial cells in normal male embryos, and we are currently pursuing a series of experiments to test this possibility.

The formation of the embryonic mammary gland is a classic example of inductive development involving epithelial-mesenchymal interactions (Sakakura, 1991; Cunha, 1994). Both the formation of the mammary bud and the initial round of branching morphogenesis appear to be critically dependent on a series of reciprocal and sequential signals exchanged between the

mammary epithelium and the dense mammary mesenchyme (Thesleff et al., 1995; Cunha, 1994; Cunha et al., 1995; van Genderen et al., 1994; Weil et al., 1995, Yang et al., 1995). Several experiments have suggested that the presumptive mammary epithelium plays an important role in promoting the condensation and formation of the dense mammary mesenchyme (van Genderen et al., 1994; Kratochwil et al., 1996, Thesleff et al., 1995). However, once formed, the mammary mesenchyme appears to direct the formation of the mammary epithelial duct structure as well as to contribute to mammary epithelial cell cytodifferentiation. For example, heterotypic recombination experiments have demonstrated that mesenchymal cells from the fetal mammary gland can induce non-mammary epithelial cells to form mammary ducts and to make milk proteins (Cunha et al., 1995) and can even induce the formation of mammary bud-like structures from the epidermis of non-mammalian species (Propper, 1973; Propper and Gomot, 1973). Likewise, recent studies have demonstrated that signals derived from mesenchymal cells are important in regulating the overall rate of ductular proliferation as well as the pattern of branching that occurs during the process of branching morphogenesis (Yang et al., 1995; Jones et al., 1996; Witty et al., 1995; Phippard et al., 1996; Friedmann and Daniel, 1996). We have demonstrated that during embryonic mammary development, PTHrP gene expression is limited to the mammary epithelium while PTH/PTHrP receptor gene expression is restricted to the mesenchyme. In the context of the phenotype discussed above, these findings suggest that PTHrP acts as an epithelial message that must be received by the mammary mesenchyme in order for it to support branching growth.

Although mammary development does not appear to be abnormal in the PTHrP knockout embryos until E15-16, we have found that the PTHrP and the

PTH/PTHrP receptor genes appear to be expressed in the mammary bud from its formation, at E12, onward. Furthermore, our K14 transgene crossing experiment suggests that PTHrP is dispensable before E15. K14 expression does not appear before this point, and therefore the mammary epithelium in the K14-PTHrP/PTHrP-null mice does not make PTHrP before E15. Despite this delay in PTHrP secretion, as compared to normal mice, K14-PTHrP/PTHrP-null mice successfully initiate branching growth of the mammary ducts. This would imply that the critical period of PTHrP signaling in the mammary mesenchyme is just before the initiation of the primary growth spurt at E15 - 16. Alternatively, it is possible that any prior exposure to PTHrP might allow the mesenchyme to be competent to support the initiation of branching morphogenesis at E16. Future study of the effects of PTHrP on mammary mesenchymal cells should help to clarify the details of the temporal requirements for PTHrP signaling during embryonic mammary development.

In summary, we have found that during embryonic mammary gland development PTHrP is a necessary participant in the epithelial-mesenchymal interactions leading to the formation of the rudimentary epithelial duct system. Specifically, PTHrP is produced by the mammary epithelium and appears to act on the mesenchyme, allowing it to support the initiation of branching morphogenesis. We have previously reported that the overexpression of PTHrP in mammary myoepithelial cells had dramatic effects on the process of branching morphogenesis during sexual maturation and pregnancy (Wysolmerski et al., 1995), indicating that PTHrP likely plays an important role in the regulation of this process throughout mammary development. There is also growing evidence of the participation of PTHrP in the reciprocal epithelial-mesenchymal interactions that govern epithelial development in sites other than the mammary gland. For example, the pattern of epithelial PTHrP expression

and mesenchymal PTH/PTHrP receptor expression seen in the developing mammary gland has been noted in other developing organs whose morphogenesis relies on epithelial-mesenchymal interactions (Lee et al. 1995). In addition, as described in the companion manuscript, Philbrick et. al. (1997) have demonstrated that col II-PTHrP/PTHrP-null mice have multiple defects in ectodermally-derived organs that are dependent on epithelial-mesenchymal interactions for their development. We anticipate that PTHrP will be found to participate in the regulation of mesenchymal cell function during the development of a number of epithelial organs, and it is our hope that further study of the effects of PTHrP during embryonic mammary development will provide a framework for the general understanding of PTHrP's role in regulating mesenchymal function during organogenesis.

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Figure Legends

Figure 1. Comparison of the embryonic mammary glands of PTHrP-knockout and normal littermate embryos at E15 and E18. The ventral epidermis was dissected from the respective embryos and photographed under low magnification using transmitted light in order to examine the gross structure of the embryonic mammary glands. In A & B, the mammary buds appear as round structures projecting upwards from the undersurface of the epidermis (arrowheads). Note that at E15 the mammary buds in the knockout embryos appear similar to those in the normal embryos. In contrast, by E18 there is a dramatic difference in the appearance of the mammary structures in knockout (E & F) as compared to normal (C & D) embryos. At this point, the normal mammary structure consists of a developing nipple (dark halo, arrow in C) and an elongated primary duct (translucent tube-like structure between arrowheads in C & D) which is just beginning to form initial branches as it makes contact with the developing mammary fat pad (labeled F.P.). Note that in the PTHrP-knockout embryos (E & F), the mammary glands fail to elongate and remain bud-like (E) or slightly ectatic (F) in their appearance. There are no primary ducts that extend to the fat pads, and the developing fat pads (F.P.) themselves appear diminished in size. Scale bar represents 160 microns for all panels.

Figure 2. Histologic comparison of the embryonic mammary glands of PTHrP-knockout and normal littermate embryos at E15 and E18 . A & B are photomicrographs of H&E-stained sections through mammary buds dissected from a normal littermate (A) and a PTHrP-knockout (B) embryo at E15. At this stage, the mammary bud consists of an invagination of mammary epithelial cells surrounded by a condensation of mammary mesenchyme, and

the microscopic appearance of the PTHrP-knockout buds was entirely normal. C through G are photomicrographs of H&E-stained sections through mammary glands dissected from PTHrP-knockout (E & G) and normal littermate (C,D & F) embryos at E18. In a normal embryo (C & D) one can see the primary epithelial duct (arrowhead in C) arising from the epidermis and extending below the dermis where it branches (arrowheads in D) and makes contact with the preadipocytes (arrows in D) within the developing fat pad. In contrast, in the PTHrP-knockout embryos (E) the epithelial duct (arrowheads) does not extend out of the upper regions of the dermis and becomes surrounded by an abnormally dense condensation of fibroconnective tissue (arrow in E). F & G are high-power photomicrographs of mammary epithelial ducts in crossection taken from a normal (F) and PTHrP-knockout (G) embryo at E18. Note that in the knockout duct (G) the epithelial cells appear to be degenerating; many nuclei are pycnotic, the cell cytoplasm appears reduced and somewhat vacuolated and the cells are separating from the basement membrane. Scale bar represents 16 microns in A&B, 25 microns in C&D, 17 microns in E and 5 microns in F&G.

Figure 3. Histologic determination of apoptosis in embryonic mammary ducts of PTHrP-knockout and normal littermate embryos at E18. TUNEL assay performed on sections of mammary ducts taken from normal (A) and PTHrP-knockout embryos (B) at E18. Note the large number of apoptotic cells (bright green nuclei) in the central portion of a normal duct (borders outlined by arrowheads) in A. In contrast, there are no positively-staining cells in the knockout ducts (outlined by arrowheads) in B. Despite the lack of positive cells in the mammary ducts in the knockout embryos, cells in the

upper layers of the epidermis continued to stain (see arrow), as was the case in normal embryos (not shown). Scale bar represents 17 microns.

Figure 4. Histologic comparison of the embryonic mammary glands of PTH/PTHrP receptor-knockout and normal littermate embryos at E 18. A. Photomicrograph of H&E-stained sections of mammary rudiment from a normal littermate. Note the initial branches of the primary duct (arrowheads) within the lower dermis. Also, note the developing nipple sheath (arrow). B. Photomicrograph of H&E-stained sections of mammary rudiment from PTH/PTHrP receptor-knockout embryo. Note that the mammary duct has not elongated, that the mammary rudiment remains bud-like in its appearance and that there is no nipple sheath. C. Higher magnification of B. Note that the epithelial cells appear to be degenerating; there are many pycnotic nuclei and the cell borders are indistinct, similar to the appearance of the PTHrP-knockout epithelial cells at this time point. D & E. H&E-stained crosssections of epithelial ducts from normal (D) and PTH/PTHrP receptor-knockout (E) mammary glands at E18. Note the lacy, delicate appearance of the stroma surrounding the normal ducts (D) as they make contact with the mammary fat pad. In contrast, note the condensation of stroma surrounding a rare PTH/PTHrP receptor-knockout duct (E) that has attempted to grow out from the mammary bud. Scale bar represents 20 microns in A&B, 4.5 microns in C and 10.4 microns in D&E.

Figure 5. Localization of PTHrP and PTH/PTHrP mRNA expression in normal embryonic mammary glands. A-C. In situ hybridization for PTHrP mRNA in normal mammary rudiments at E16. A & B are brightfield and darkfield images, respectively, of the same section hybridized with antisense probe. C is a brightfield image of a similar section hybridized to sense probe as

a control. Note that PTHrP mRNA is found in the mammary epithelial cells, especially those located peripherally. There is no hybridization within the mesenchyme. Note the lack of hybridization of the sense probe (compare A & C). D-F. In situ hybridization for PTH/PTHrP receptor mRNA in normal mammary rudiments at E15. D & E are brightfield and darkfield images, respectively, of the same section hybridized with antisense probe. Note that PTH/PTHrP receptor mRNA is found within the dense mammary and dermal mesenchyme; there is no receptor mRNA expressed within the mammary epithelial cells. F is a brightfield image of a similar section hybridized to PTH/PTHrP receptor sense probe as a control. Note the lack of signal as compared to D. Scale bar represents 15 microns for all panels.

Figure 6. Immunohistochemical analysis of keratin-14 (K-14) expression during normal embryonic mammary development. A. Histologic section through a mammary bud from a normal embryo at E13, stained for K14 (brown) and counterstained with hematoxlyn (blue). B. Section through a mammary bud at E15 stained as above. C. Section through a mammary epithelial duct from an embryo at E18, stained for K-14. Note that appreciable amounts of K14 are detectable at E15 but not at E13. Also, note the basal location of K14 staining in the epidermis, as has been reported (Kopan and Fuchs, 1989). Finally, note that, at these embryonic time points, K14 appears to be expressed uniformly by all mammary epithelial cells. Scale bar represents 12.5 microns for all panels.

Figure 7. Mammary development in K14-PTHrP/PTHrP-null mice. A. & B. are photographs of the whole mammary glands of a normal (A) and K14-PTHrP/PTHrP-null neonate. The ventral skin was dissected and the mammary

glands were viewed under low magnification using transmitted light. In both cases, the mammary gland consists of a primary duct (between arrowheads) that forms several branches before entering the mammary fat pad (dark area to the left). (Contrast B with the lack of a primary duct in the original PTHrP-null embryos as shown in Fig. 1 E & F.) C. is a photomicrograph of an H&E-stained section through the mammary gland of a K14-PTHrP/PTHrP-null neonate. Note that the primary duct extends from the epidermis through the dermis and forms its initial branches (arrows), as occurs in normal mammary development. However, despite the rescue of mammary epithelial development, note the lack of a nipple sheath. Scale bar represents 67 microns in A&B and 17 microns in C.

Figure 1

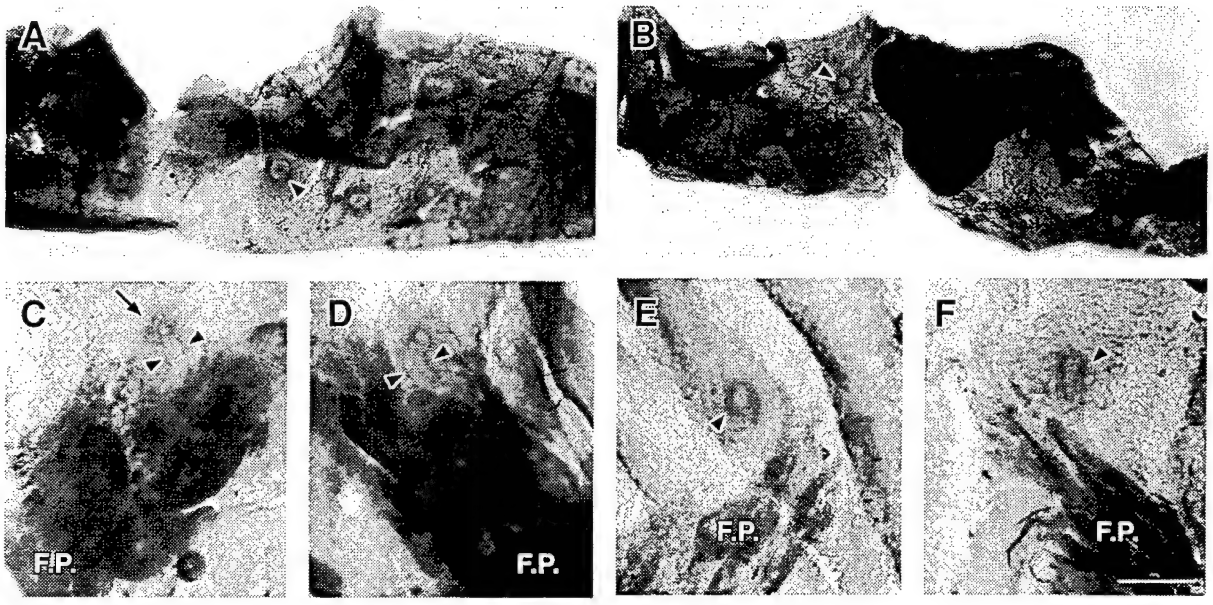
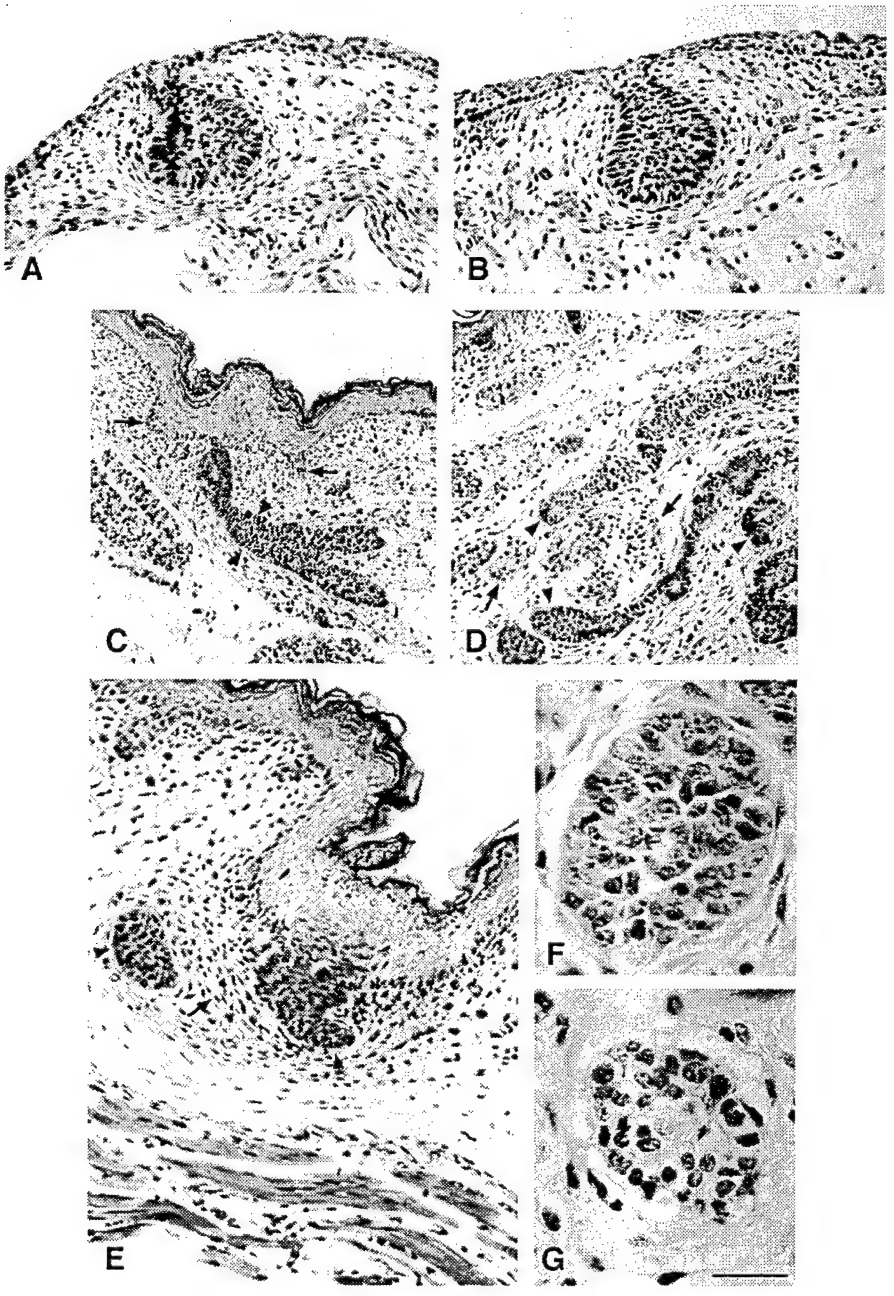


Figure 2



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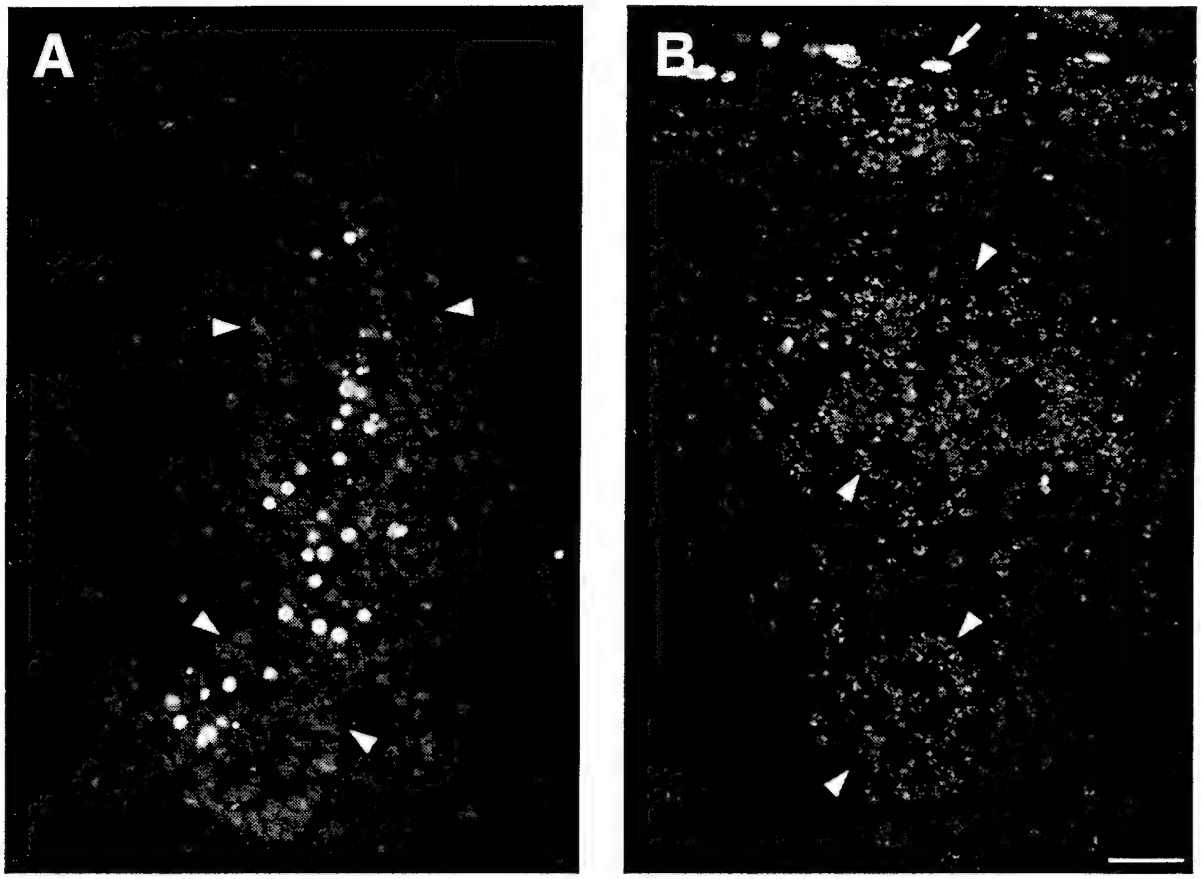

Figure 4

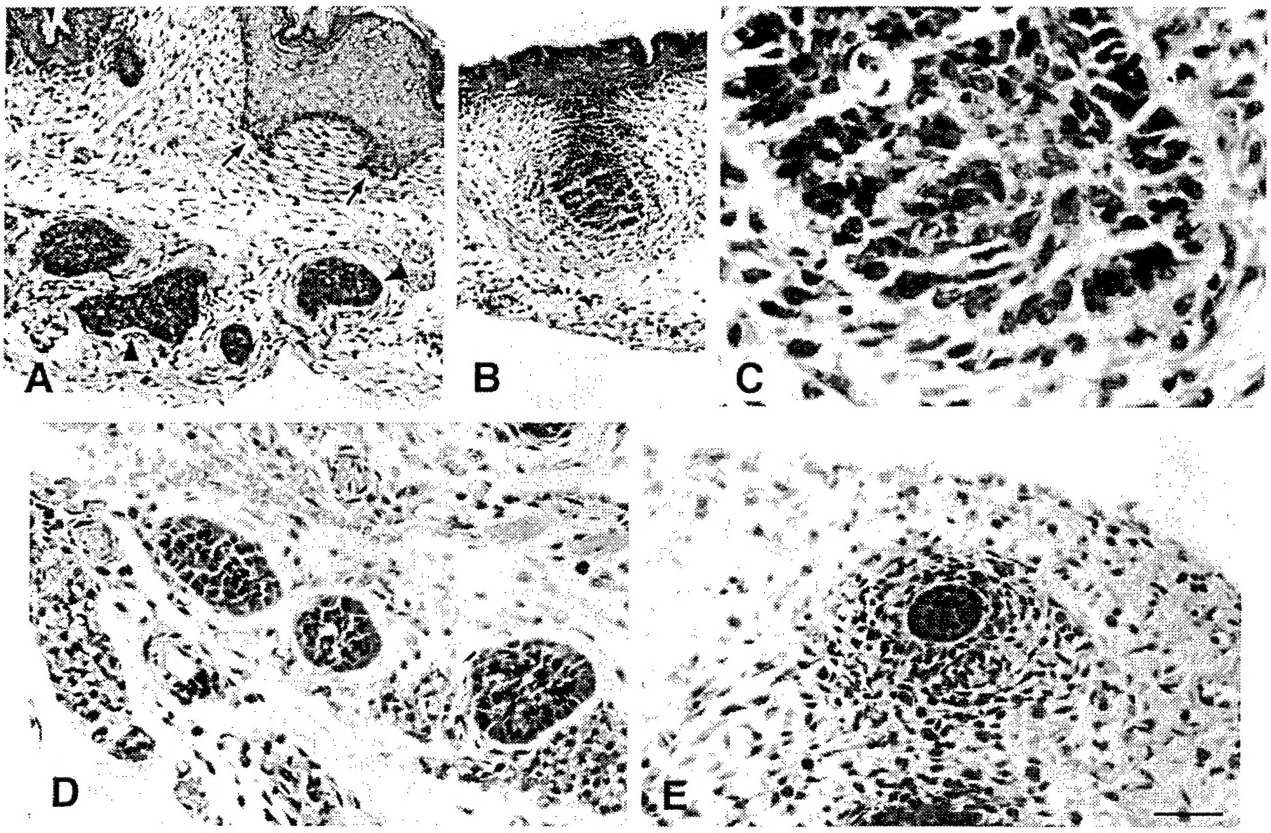

Figure 5

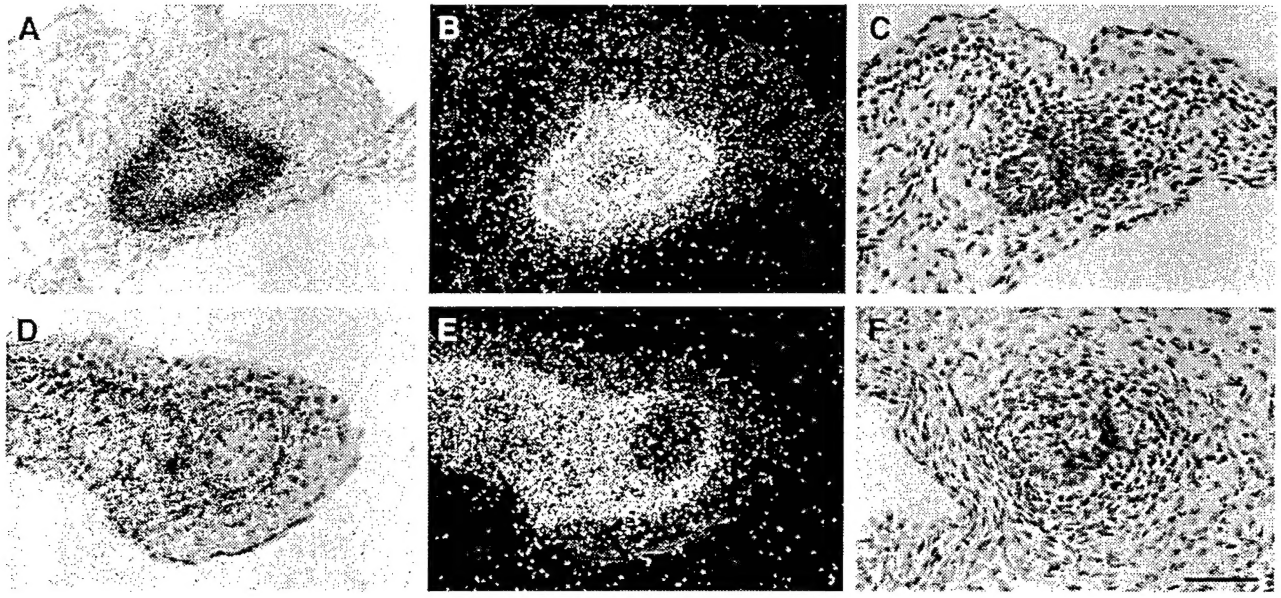

Figure 6

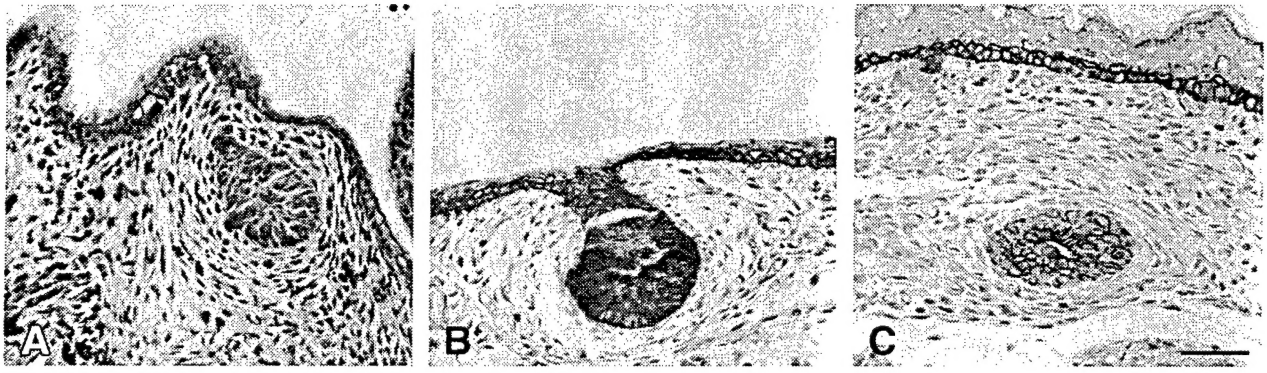

Figure 7